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The role of potential antioxidant in medicinal drug-induced oxidative stress

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THE ROLE OF POTENTIAL ANTIOXIDANT IN MEDICINAL DRUG-INDUCED OXIDATIVE STRESS

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ABSTRACT

Most medicinal drugs have adverse effects. Among the most commonly used of these drugs are several types, known as "oxidative drugs". These are believed to cause adverse effects that induce oxidative stress, an imbalance of generation and detoxification of reactive oxygen species. So it is a reasonable assumption that the antioxidant might alleviate the toxicity induced by these drugs. N-acetylcysteine (NAC), a synthetic thiol, is a free-radical scavenger and a precursor of glutathione (the main endogenous antioxidant). However, the negative charge of NAC at physiological pH limits its bioavailability. N-acetylcysteineamide (NACA) is neutral in charge and is believed to have higher lipophilicity than NAC. Experiments were designed to determine the potential protective ability of NACA against different oxidative drugs. Positive results from our studies show that NACA seems to be a broad-spectrum protector that prevents oxidative damage. This underscores NACA's potential for serving as a clinical protective antioxidant for patients who receive potentially dangerous oxidative drugs.

Sutherlandia frutescens (SF), a legume native to South Africa, is a historical herb medicine for treatment of a variety of disorders. It is believed that SF therapeutic effects are at least partially due to its antioxidant potential. Cell-free studies were conducted for reducing power, radical-scavenging power and metal-chelating capacity of SF extract, as well as *in-vitro* study for its possible protective role against exogenous oxidative stress in three cell lines. Positive results from our preliminary studies further verified the antioxidant effect of SF, indicating its promising future as an antioxidant supplementation for clinical purpose.

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1. INTRODUCTION

"Adverse Effect", in the field of pharmacy, refers to the undesired deleterious impact of certain medications on a patient. This can be seen upon administration of almost any type of medicinal drug. The underlying mechanisms that promote these adverse effects were investigated by a pharmacologist and a toxicologist to determine a way to diminish there severity or to eliminate any undesired effects.

Upon clinical application, several types of medicinal drugs were found to cause severe hematotoxicity (i.e., acute hemolysis) in individuals with certain genetic disorders, among which the most common is glucose-6-phosphate dehydrogenase deficiency (G-6-PDD). These drugs, upon further studies of the mechanisms of their adverse effects, were found to be hematotoxic because they cause oxidative stress by generating excessive reactive oxygen species (ROS) and/or disrupting the endogenous antioxidant defense system. They are collectively referred to as "oxidative drugs".

The most common "oxidative drugs" include: (1) several antipyretic-analgesic drugs (mainly NSAIDs), such as aspirin, phenacetin, and acetaminophen (Tylenol); (2) most sulfonamide antibiotics, such as sulfamethoxazole, dapsone, or sulfadiazine; (3) nitrofurans, such as nitrofurantoin; (4) some anti-tuberculosis drugs, such as isoniazid; (5) some anti-malarial drugs, such as quinine and primaquine; (6) alkylating agents, such as busulfan and cyclophosphamide; (7) anthracyclines, such as doxorubicin.

Today, "oxidative drugs" are usually contraindicated in patients with the mentioned genetic disorders due to the high risk of a life-threatening hemolytic reaction. Nevertheless, these drugs also commonly induce dose-dependent hematic, pulmonary,

hepatic, or renal toxicities in patients of normal genotype, especially upon continuous administration and/or an overdose. For example, a life-threatening acetaminophen overdose alone could cause ~56,000 emergency department visits in the U.S. annually.

The severity of adverse effects, resulting from oxidative drug administration, may vary from being mildly uncomfortable to fatal complications, such as acute hepatic failure upon an overdose of acetaminophen or other analgesics. In certain cases, severe adverse effects can become the main concern of physicians who may resort to planning a therapeutic regimen and even restrict or prevent the administration of certain drugs. For example, this may be needed in cases such as the life-threatening adverse effect of pulmonary fibrosis induced by bleomycin. Accordingly, there is a pressing need for effective strategies that can:

Minimize the side effects of therapeutic doses of oxidative drugs to improve the drug tolerance of patients, especially during cancer treatment;

Alleviate or reverse the toxicity of an overdose of an oxidative drug.

It is a reasonable hypothesis that the administration of an antioxidant might be a solution for oxidative-drug induced toxicity. However, another main concern is that an antioxidant might diminish the desired effect of the medicinal drug, especially when the drug exerts its primary effect via ROS-related pathways. Therefore, it is critical to unravel details about the mechanisms that result in desired and adverse effects of oxidative drugs, as well as the possible roles an antioxidant might play in co-administration.

In human body, there are endogenous antioxidant compounds/enzymes, altogether serving as a defending system against oxidative damage. Γ-L-Glutamyl-L-cysteinylglycine, commonly known as glutathione (GSH), is the primary cellular antioxidant that reduces or conjugates with pro-oxidant species, as well as chelates transition metal ions which catalyze Fenton reaction, thereby detoxifying ROS and repairing oxidative damages. Direct administration of GSH, however, is proven not applicable due to presence of γ-glutamyltransferase in most tissues, which hydrolyzes GSH. Therefore, substitute antioxidants that might favor GSH repletion are taken into consideration.

N-acetylcysteine (NAC), a synthetic derivative of L-cysteine, is a non-toxic precursor of GSH de-novo synthesis. NAC is FDA-approved as an antidote of acetaminophen overdose, due to its antioxidant potential. Bioavailability of NAC, however, is limited due to the negative charge on its carboxylic group at physiological pH. N-acetylcysteineamide (NACA), the amide form of NAC, has been found to possess higher antioxidant potency compared to NAC, possibly because its electrical neutrality increases its lipophilicity and bioavailability. In this study, the in-vitro antioxidant effects of NAC and NACA against oxidative stress induced by medicinal drugs were comparatively evaluated. The results my help develop a novel strategy in alleviation of oxidative-stress-related adverse effects of medicinal drugs, as well as promote future clinical substitution of NAC with NACA.

Except synthetic compounds, plant-derived natural antioxidants are consistently highly interested due to their high efficacy and low toxicity. *Sutherlandia frutescens,* a legume native to South Africa, is historically used as herb medicine. It is believed that its

therapeutic effect is at least partially due to its antioxidant potential. Therefore, *S. frutescens* might be a candidate for treatment of adverse effects oxidative medicinal drug. For purpose of preliminary study, experiments were conducted for radical scavenging power of *S. frutescens* extract in cell-free system, as well as its in-vitro protective effect against *tert*-butyl hydroperoxide (tBHP). The results might help in comprehensive evaluation of antioxidant effect of S.frutescens as a potential treatment for oxidative stress induced by medicinal drugs.

2. REVIEW OF LITERATURE

"Oxidative drug" is a collective term, as mentioned above. Different types of oxidative drugs induce oxidative stress via distinct mechanisms. In the following literature review, therefore, oxidative drugs are categorized into groups based on the mechanism of oxidative stress induction. Mechanisms of the adverse effects of these drugs were discussed; also, results are presented from previous studies about the roles of a co-administered antioxidant.

2.1. OXIDATIVE STRESS: CYP-450 MEDIATED MECHANISM

There are several groups of compounds that, although they induce oxidative stress in the human body, they do not manifest oxidative activity per se. Instead, they are metabolized to toxic intermediates by CYP450-dependent enzymes in the liver and/or by other enzymes in various tissues in the human body. These highly active intermediates directly attack essential biomolecules (such as DNA, proteins, and lipid), causing oxidative damage, or conjugate with reduced glutathione (GSH) via enzymatic or non-enzymatic reactions due to their electrophilicity. They are then excreted, depleting intracellular GSH (especially from the liver, where the vast majority of detoxification reactions occur), and rendering the body's cells vulnerable to oxidative damage.

Drug metabolism refers to the process of biological modification of xenobiotics by organisms, during which foreign compounds are generally converted to more hydrophilic products to be readily excreted. Although these modification reactions mainly act to detoxify the compounds, there are many cases in which the intermediate or product of xenobiotics are more toxic, compared to the original compounds.

The drug metabolism process primarily occurs in the liver and kidneys. The process could be divided into three phases: primary modification, secondary conjugation, and excretion. In phase I, the reactive and polar functional groups, such as the epoxy, carbonyl, hydroxyl, sulfhydryl, and amine groups, are introduced in nonpolar hydrocarbons via catalysis of the hepatic CYP450-dependent mix function oxidase/reductase, or enzymes in other systems. These intermediates usually further undergo phase II metabolism and are detoxified and excreted; however, many of them are more reactive and toxic than the initial substrate and if, not eliminated rapidly, might react with neighboring bio-molecules to cause damage or form an antigen complex and initiate immunoreaction.

In phase II, these activated primary metabolites conjugate with small endogenous molecules, so that hydrophilicity is further increased in favor of rapid excretion. The type of molecules conjugated depends on the chemical properties of the primary metabolites. Those bearing epoxy, carbonyl, or other electrophilic functional groups will be conjugated with nucleophilic molecules, such as GSH or other compounds with sulfhydryl groups, whereas those bearing hydroxyl, sulfhydryl, amine, or other nucleophilic groups are prone to sulfation, acetylation, or glucuronidation. Generally, the products of phase II metabolism are of reduced reactivity and are ready to undergo renal excretion. However, in cases of excessive phase I primary metabolite (such as an acetaminophen overdose, which will be discussed in a subsequent section), phase II metabolism could result in depletion of GSH and, therefore, make the cell vulnerable to oxidative stress.

CYP450-enzyme mediated metabolism partially or primarily accounts for oxidative stress induced by most oxidative drugs, including antipyretic/analgesic drugs, sulfonamide antibiotics, alkylating anti-tumor drugs, and isoniazid, which will be briefly introduced in the following section. Although "traditional" antimalarial drugs (such as primaquine), require CYP-450 mediated oxidation for activation, they exert oxidative toxicity via a redox cycle of their metabolite to generate "secondary" reactive oxygen species. This will be introduced in a separate section later.

2.1.1. Antipyretics and Analgesics – NSAIDs and Acetaminophen. Non-steroidal anti-inflammatory drugs (NSAIDs) in chemistry collectively refer to a class of drugs that are not steroids, but they exert antipyretic, analgesic, and anti-inflammatory effects. Acetaminophen (Tylenol), although not considered a NSAID, because of its minimal anti-inflammatory effect, will be discussed in this section as representative of NSAIDs due to its similar mechanism that affect desired as well as side effects.

NSAIDs exert their therapeutic effects via inhibition of cyclooxygenases (COXs), which are a group of isozymes that converts arachidonic acid into prostaglandin and thromboxane, a subclass of eicosanoids that mediate the sense of pain, inflammation, and thermoregulation, and induce blood coagulation (platelet aggregation), and vasoconstriction (or vasodilation, depending on the type of receptor), as well as constriction of other smooth muscles.

There are two major types of COXs. COX-1, generally referred to as "physiological COX", is a ubiquitous and constitutively expressed enzyme that plays an essential role in many normal processes, one of which is stimulating the secretion of

gastric mucus as well as inhibition of excessive secretion of gastric acid; whereas COX-2, referred to as “pathological COX”, is used to express mediation of inflammation. Therefore, it is inhibition of COX-2 that produces the desired effects of NSAIDs. However, “old” NSAIDs, such as aspirin, indomethacin, or ibuprofen, unselectively inhibit both COXs and, therefore, induce or exacerbate gastric ulcer/bleeding and disorders in blood coagulation. The new NSAIDs, as well as acetaminophen, selectively inhibit COX-2 to exert antipyretic/analgesic functions with minimal side effects, discussed above.

Acetaminophen is one of the most common over-the-counter drugs in the United States. According to Lee et al. [3] Tylenol alone produces over 1 billion USD for sale annually in the U.S. With a therapeutic dosage, acetaminophen is considered rather safe; however, a considerable number of cases of acetaminophen overdose, unintentional or intentional (suicidal), are still reported throughout the country every year. Statistically, there are more than 100,000 calls to Poison Control Centers annually due to an acetaminophen overdose, as well as over 56,000 Emergency Room visits, with an average of 2,600 cases requiring hospitalization. Of those hospitalized, about 460 patients die due to acute liver failure annually,.

As for the mechanism of the therapeutic effect of acetaminophen, a highly selective COX-2 inhibitor, especially COX-2 in the central nervous system which prevents generation of prostaglandin E2 and exerts its antipyretic and analgesic effects. However, due to its selectivity, acetaminophen has a minimal anti-inflammatory function, nor is it an anti-coagulant, unlike aspirin. Although it is not counted as a typical member

of NSAIDs, acetaminophen is still frequently studied, along with and compared to NSAIDs due to their similar mechanisms.

The main adverse effect of an overdose of acetaminophen is its acute hepatic and renal toxicity, which may result in hepatic/renal failure and death of the patient, in extreme cases. The hepatotoxicity of acetaminophen are well studied and confirmed in both in-vitro and in-vivo models. Now, the best-known and most commonly-accepted mechanism is that acetaminophen, upon an overdose, is converted to N-acetyl-p-benzoquinone imine (NAPQI) by hepatic cytochrome enzyme P450 (CYP450) and, thereafter, depletes intracellular glutathione, resulting in oxidative stress and subsequent necrosis of hepatocytes.

Under physiological conditions, acetaminophen is mainly metabolized in the liver via glucuronidation (approximately 40%) and sulfation (20-40%) by UDP-glucuronyltransferase and sulfotransferase, respectively, in favor of further excretion; [5] whereas only ~15% is oxidized by CYP450 isozymes (mainly CYP2E1, 1A2, and 2D6) to NAPQI, an electrophilic and highly active intermediate, which are conjugated with reduced glutathione at the sulfhydryl group either non-enzymatically or via catalysis of glutathione S-transferase (GST), to form 3-(glutathione-S-yl) acetaminophen.[6] With an overdose, however, enzymes for both the glucuronidation and sulfation pathways are saturated and a significant amount of NAPQI is generated, which is responsible for acetaminophen hepatic toxicity. Actually, the hypothesis that either CYP2E1-knockout or CYP450 inhibitor would significantly reduce acetaminophen toxicity is supported by experimental results [7]. The mechanism of APAP toxicity is schemed in Figure 2.1.

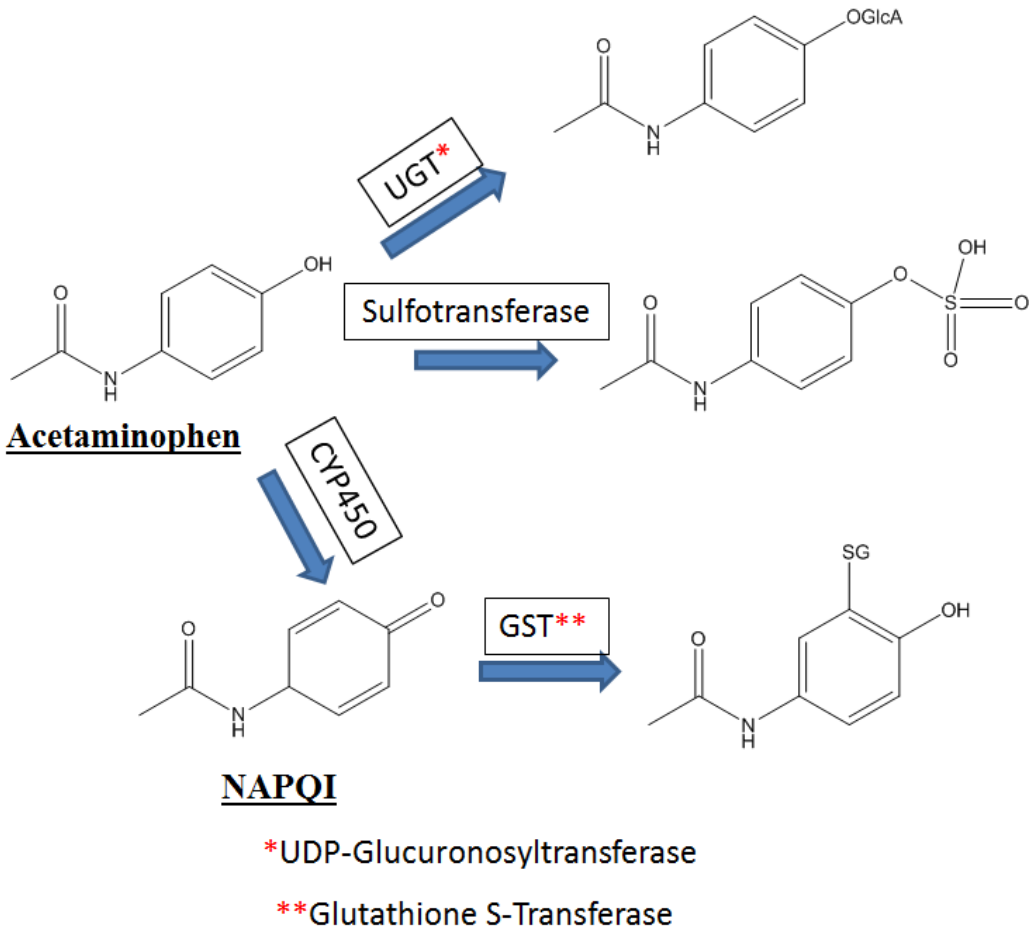

Figure 2.1. Metabolism of APAP. At therapeutic dose, acetaminophen is primarily glucoronidated or sulfated and excreted. However, upon overdose, excessive acetaminophen is mediated by cytochrome P450 isozymes, producing N-acetyl-p-benzoquinone imine (NAPQI), which is conjugated with glutathione and excreted, therefore depleting intracellular glutathione.

NAPQI results in acetaminophen toxicity via several pathways, among which the most commonly known is that, upon depletion of GSH (a 90% decrease in the intracellular GSH level), highly electrophilic NAPQI molecules attack and bind to protein-bearing sulfhydryl groups to form acetaminophen-protein adducts. This results in dysfunction of the corresponding proteins. Actually, a number of proteins that form an

adduct with NAPQI have been isolated, purified, and identified by sequence analysis or mass spectroscopy studies [8]. This includes various enzymes that are normally expressed in cytosol (such as N-10-formyltetrahydrofolate dehydrogenase), mitochondria (such as glutamate dehydrogenase), and microsome (such as glutamine synthetase), indicating wide intracellular distribution and damage to NAPQI. Specifically, conjugation of NAPQI with mitochondrial enzymes causes a series of devastating consequences. It causes disruption of mitochondrial membranes and, therefore, mitochondrial permeability transition (MPT), the phenomenon that occurs when mitochondrion loses its impermeability and results in leakage of molecules lower than 15,000 Dalton (including cytochrome c), which could trigger apoptosis (once present in cytosol) and out-leakage of calcium ions. Besides, it also results in dysfunction of the electron-transport chain (ETC) on the mitochondrial inner membrane results in (1) decrease in ATP production; (2) "premature" leakage of electrons to oxygen molecules and generation of excessive superoxide anion, which is considered to be a source of "secondary oxidative stress" in acetaminophen-induced toxicity. Actually, superoxide anion over-production has been confirmed [9] by experimental results that show that the activity of aconitase (an enzyme typically inhibited by a superoxide ion) is due to the sensitivity of its iron-sulfur cluster that decreases upon acetaminophen dosing in human macrophages.

Acetaminophen-SG, the conjugative product of acetaminophen and reduced glutathione, has long been considered to be non-toxic. In a recent study, [10] however, purified acetaminophen-SG was tested for its toxicity to rat liver mitochondria and was found to induce the generation of ROS upon co-incubation with either complex I or II substrates. This indicated that complex I and II could be the main mitochondrial enzymes

that are impaired by acetaminophen-SG dosing and cause the generation of ROS via premature electron leakage.

Peroxynitrite toxicity could be an indirect consequence of GSH depletion resulting from an acetaminophen overdose. Peroxynitrite, the product of additive reaction between nitric oxide and superoxide anion, could not only directly attack biomolecules as a reactive nitrogen species (RNS) but also cause damage by the formation of cytotoxic nitrotyrosine. Normally, peroxynitrite is detoxified via the GSH/GSH peroxidase (GPx) pathway, which is impaired due to depletion of the substrate by an acetaminophen overdose.

As for the role of GPx in mediating acetaminophen toxicity, however, there are some paradoxical results [11]. It was reported that over-expression of superoxide dismutase (SOD) or serum GPx would increase the resistance of experimental animals towards acetaminophen toxicity; however, an increase in intracellular GPx levels sensitized the animals. It was assumed that the reaction, catalyzed by intracellular GPx, exacerbated GSH depletion and primarily resulted from NAPQI conjugation. However, further study is necessary for elucidation of the exact mechanism.

The other possible route, by which secondary ROS/RNS is generated, is that hepatic macrophages or other innate immunocytes are recruited and activated (triggered either directly by necrotic hepatocytes or due to up-regulation of several inflammatory factors) to produce superoxide or other reactive species. Actually, it has been reported that suppression of Kupffer cell (hepatic macrophages) activity desensitized the animals to acetaminophen toxicity. [12]

In some cases, oxidative enzymes other than CYP 450 (such as NADPH oxidase or myeloperoxidase) can also oxidize and activate acetaminophen and further exacerbate oxidative damage. Some in-vitro studies of rat liver microsomes were conducted to elucidate the enzymatic pathways of acetaminophen activation when CYP450 and NADPH oxidase are available, since both are capable of generating ROS through the metabolization of acetaminophen with NADPH as a substrate. [13] It was found that selective NADPH oxidase reversed the trend of increased lipid peroxidation and decreased the activity of GST upon acetaminophen dosing, but not that of decreased intracellular GSH level, whereas CYP 450 inhibitor prevented all alterations. It was thus confirmed that CYP450 was involved in acetaminophen activation and toxicity in rat liver microsomes to some extent, but was not the major contribution.

One of the main adverse effects of acetaminophen on a CYP-2E1-mediated mechanism is renal toxicity, which is similar to the adverse effects of hepatic toxicity. [14] It was found that an acetaminophen overdose resulted in proximal tubular necrosis in humans, rats, and mice. The weight of the kidney as well as the blood urea nitrogen (BUN) both increased. In kidneys, it was found that certain proteins--27, 33, 56, 58kDa--were predominantly arylated and conjugated with acetaminophen, resulting in their dysfunction.

2.1.2. Sulfonamide Antibiotics. Sulfonamides are a group of traditional antibiotics that have been used in clinics since the 1930s. They were the most commonly used antibacterial drugs before the discovery of natural antibiotics such as penicillin. Today, sulfonamides have rekindled the interest of physicians and pharmacists due to their effect against both Gram-positive and Gram-negative bacteria, as well as the

concern about the increasing tolerance of "super" bacteria strains against traditional natural antibiotics. It is noted, however, that of all prescribed antibiotics, sulfonamides cause the most cases of emergency room visits, usually due to hypersensitivity reactions. The most commonly used sulfonamide antibiotics include sulfamethoxazole (SMX), sulfadiazine, and dapsone (DDS) (structures shown in Figure 2.2. and Figure 2.3.). Their toxicity has been widely studied in a variety of cell models in in-vitro studies, as well as in in-vivo studies, and will be discussed in later sections.

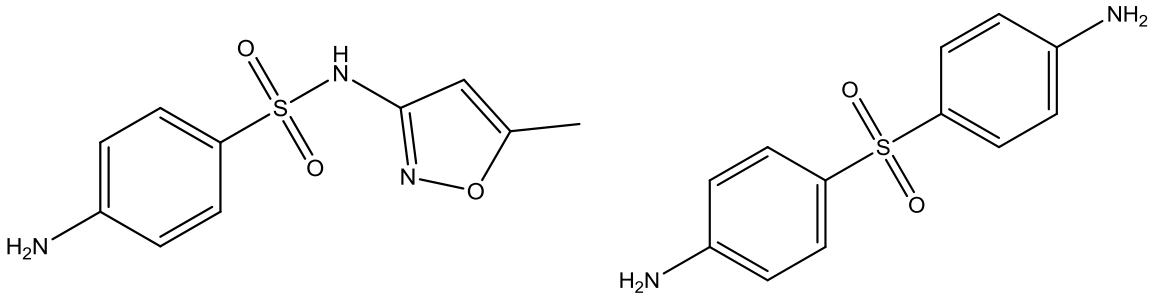

Figure 2.2. Comparison of SMX and DDS structures. There is one amine group *para* to sulfonyl group in SMX molecule, and two in DDS molecule.

The antibiotic effects of sulfonamides mainly result from their structural similarity to para-aminobenzoic acid (PABA), which is one of the substrates involved in bacterial de-novo biosynthesis of tetrahydrofolic acid, a pivotal co-factor for a variety of essential bioreactions. In the first step of tetrahydrofolic acid synthesis in prokaryotes, PABA conjugates with 2-amino-4-hydroxy-6-pyrophosphoryl-methylpteridine to form dihydropteroate, to which L-glutamate is further conjugated to form dihydrofolic acid. Sulfonamide mimics the structure of PABA and, therefore, could be similarly conjugated to pteridine via catalysis of dihydropteroate synthase. The sulfonamide-pteridine adduct,

however, blocks dihydrofolate synthetase, the enzyme for the second step, by (1) competitive inhibition with normal PABA-pteridine adduct; (2) non-competitive inhibition via occupation of the site which is normally occupied by L-glutamate. Therefore, bacterial biosynthesis of tetrahydrofolic acid is inhibited and bacterial cells die because of the consequent disruption of metabolism with requires tetrahydrofolic acid. Eukaryotic cells are less affected, since tetrahydrofolic acid could diffuse or be actively transported into a cell as an alternative route, other than de-novo synthesis.

The main adverse effects of sulfonamide antibiotics include (1) necrotic epidermal detachment that involves hypersensitivity reaction; (2) hemolytic anemia. These two types of seemingly distinct adverse effects, surprisingly, are of the similar mechanism in the initial stage, although the details as well as the following steps of pathogenesis are different. Both initial steps involve CYP450-mediated activation of the mother compound and covalent binding of the activated metabolite with protein (or other factors) to trigger the further reactions. The mechanism of SMX and DDS toxicity are shown in Figure 2.3.

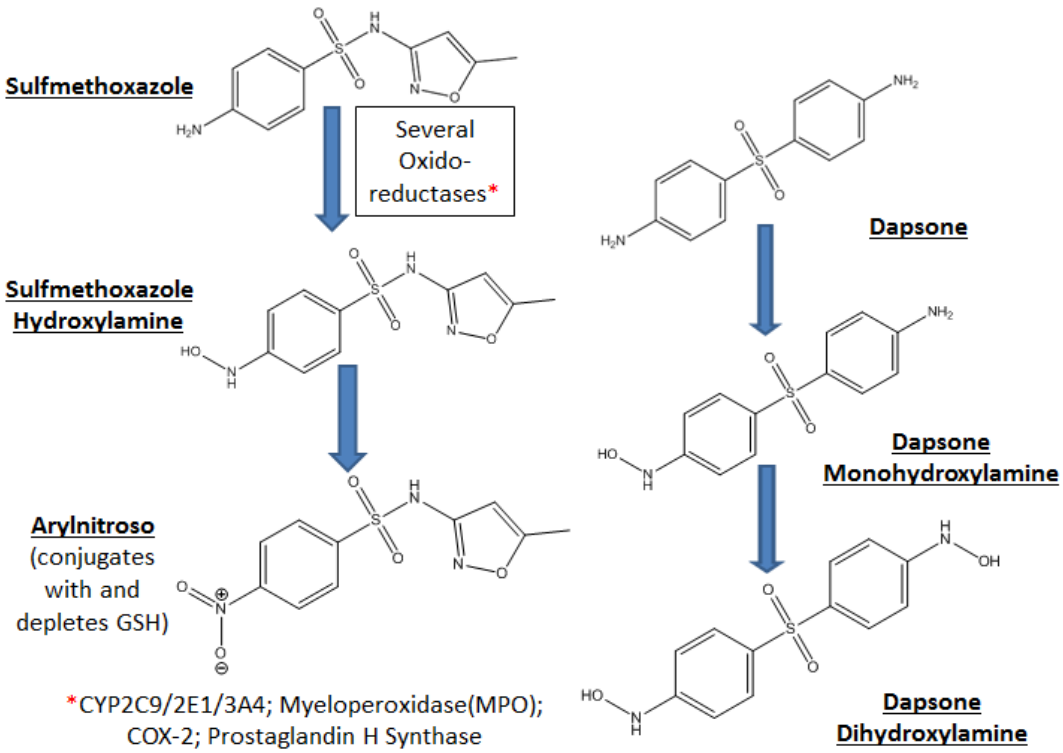

Figure 2.3. CYP-mediated metabolism of SMX and DDS. As shown in the figure, the aromatic amino groups of both compounds are first hydroxylated by a variety of oxidoreductases to hydroxylamines, which are in turn auto-oxidized to arylnitroso and conjugates with intracellular glutathione.

Upon uptake, sulfonamides follow a pathway that is similar to the CYP450-mediated metabolic pathway. CYP450 monooxygenases (or other oxidases such as COX-2, flavin-containing monooxygenases, or NAD(P)H oxidases) oxidize sulfonamide to corresponding hydroxylamine which, in turn, might be either acetylated to hydroxamic acid and undergo a series of the following reactions to be detoxified, or automatically oxidized to the corresponding nitroso. This is highly electrophilic and, therefore, may (1) conjugate with proteins or other biomolecules bearing nucleophilic functional groups; and (2) deplete GSH and directly cause oxidative stress. Furthermore, the reaction of the

auto-oxidation of hydroxylamine might couple with a partial reduction reaction of oxygen molecules to superoxide anion, which is another source of sulfonamide-induced oxidative stress.

The fate of the nitroso intermediate varies depending on the relative activity of involved enzymes in the intriguing metabolic process which, in turn, determines the type of adverse effect. The mechanisms of each in the following sections will be discussed.

The most common and severe adverse effects of sulfonamide antibiotics are hypersensitivity reactions, among which the best known ones are Steven-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). The former one could be considered a "mild subtype" of the latter (defined clinically that patients with SJS have less than 10% body surface area of skin detachment, while those of overlapped SJS/TEN have 10-30%, and those with a "typical" TEN have over 30%) [15]. SJS and TEN are life-threatening skin conditions that involve separation of the epidermis from the dermis in skin, ulcers, and other lesions in mucous membrane, as well as epidermal necrosis with minimal associated inflammation.

The underlying pathogenesis of SJS/TEN is a typical type I hypersensitivity reaction, which is antibody-mediated and only strikes a portion of individuals, i.e., "atopic individuals". Type I hypersensitivity reactions are mostly referred to as "allergic reactions" by the public, and involve common food/drug allergies. Upon uptake, an allergen is presented on the surface of dendritic cells (or other antigen-presenting cells, APCs). The APCs attract helper T lymphocytes that bear corresponding CD4 glycoproteins that combine with certain allergenic molecules on APCs. When combined,

the helper T lymphocytes are “switched on” and, in turn, secrete interleukin-4/5 to stimulate production of IgE by B lymphocytes. Also, activated helper T cells are capable of secreting other chemotactic factors, recruiting other immnocytes, and promoting further inflammatory reactions.

In cases of drug-induced SJS, hydroxylamine, the active intermediate CYP450 metabolite of sulfonamide, acts as “hapten” to conjugate with protein in host tissues to form antigen, which is thereafter presented by local dendritic cells. These APCs, in turn, produces a tumor-necrosis factor (TNF)-α and other factors to recruit CD8+ T-lymphocytes (“killer” T-cells) which, upon activation, trigger apoptosis of epidermal keratinocytes via a pathway mediated by perforin, a cytolytic protein that inserts itself through the plasma membrane, creating a pore and eventually causing lysis of the cell. The other way keratinocyte apoptosis can be triggered is when the APCs attract and activate specific helper T cells, which secrete interferon to induce expression of a Fas receptor (the “receptor of death”) by keratinocytes, as well as Fas ligands. A combination of Fas ligand/receptor will be followed by activation of the caspase system and, ultimately, apoptosis.

It had been believed that liver is the major site of conversion of sulfonamides to their corresponding hydroxylamines, which are thereafter distributed over the body, including the skin, due to its abundance in CYP450 and myeloperoxidases. However, researchers have subsequently discovered [18] that epidermal keratinocyte, per se, can metabolize the mother compounds to hydroxylamines, which further undergo autooxidation, form arylnitroso metabolites, and conjugate with protein to form the allergenic adduct. Meanwhile, the reaction of arylhydroxylamine oxidation can be

coupled with partial reduction of molecular oxygen to superoxide anions, i.e., the "secondary" ROS. These active species cause damage, not only directly, but also to the signaling pathway. In general, ROS will induce production of "dangerous" signals, such as heat-shock proteins (HSPs) and cytokines, for activation of the APCs. The mature APCs therefore express MHCs and migrate into draining lymph nodes, where they present MHCs to T-lymphocytes. The activated T cells, in turn, express a "skin-homing receptor" that is ready for "landing" in the keratinocyte layer beneath the epidermis. In detail, superoxide anions might up-regulate certain proteins on the APCs, which are stimulatory factors essential for activation and survival of T-lymphocytes, such as CD80, 83 and 86. Also, hydrogen peroxide (generated via dismutation of superoxide anion) might induce expression of TNF-α and IL-8 by APCs. Furthermore, ROS can up-regulate expression of adhesion molecules, such as E-selectin and ICAM-1, providing "landing site" for T-lymphocytes.

In this study, it was found that a variety of enzymes are capable of activating sulfonamides, including CYP2C9, 2E1, and 3A4, as well as myeloperoxidase, COX-2 and prostaglandin H synthase. Hydroxylamine (NOH) of both SMX and DDS induces concentration- and time-dependent cell death; however, DDS-NOH is proven of higher toxicity by showing lower LC_{50} in all three different types of cells tested (peripheral blood mononuclear cells, PBMC/normal human epidermal keratinocytes, NHEK/erythrocytes). The fact that DDS bears two N-4s and, therefore two equivalents of hydroxylamines can be generated, was assumed to be the underlying reason for the higher toxicity of DDS as compared to SMX. Similarly, in another article, toxic effects of both SMX- and DDS-NOH in NHEK, as well as their roles in the generation of

deleterious ROS, were studied [19]. It was concluded that both metabolites are ROS generators, but DDS-NOH produces a significantly larger amount.

Another rare but life-threatening toxic effect of sulfonamides is hemolytic anemia, which is categorized as Type II hypersensitivity reaction [20]. The most commonly known instance of this is immunoreaction due to blood type incompatibility during a blood transfusion. Cases of drug-induced hemolytic anemia, depending on the nature of the drug, involve three typical mechanisms: immune-complex mechanism, "haptenic" immune mechanism, and true autoimmune mechanism. Sulfonamide-related hemolysis falls in the second type. For "hapten" mechanism, the drug molecules work as hapten to conjugate with the protein molecules on the cell surface of erythrocyte, forming "complete" antigen to be presented by macrophages that engulfs the "modified" erythrocyte. The presentation of antigen activates the corresponding B-lymphocytes which, in turn, produce IgG or IgM to combine with the antigen. The complex subsequently activates the complement system, resulting in either the formation of a membrane attack complex ("pore" on the cell surface) or macrophage uptake and erythrocyte degradation.

In the case of SMX and DDS, they and their hydroxylamine metabolites undergo the process of enzyme-mediated protein haptenation, mainly "modifying" human erythrocyte carbonic anhydrase B&C or other components, but less likely hemoglobin. Besides the triggering the hemolytic hypersensitivity reaction, as mentioned above, "modification" and inactivation of carbonic anhydrase disrupt the osmolarity balance of erythrocytes and render them prone to rupture. Since carbonic anhydrase is the main enzyme that regulates the balance between carbonic acid, water, and carbon dioxide, as

well as proton and bicarbonate anion, this has a direct effect on the maintenance of osmotic pressure equilibrium.

Besides the hypersensitivity mechanism (which is predominant in “slow acetylators”, as discussed in the section about SJS/TEN), sulfonamide antibiotics are capable of inducing hemolytic anemia via a distinct mechanism that involves oxidative stress. This typically occurs in patients with certain genetic disorders such as G-6-PDD. The oxidative stress-related mechanisms are similar to those discussed in the SJS/TEN section above. It was hypothesized that sulfonamide and its hydroxylamine/nitroso metabolites induce oxidative stress via two main strategies: (1) partial reduction of O_2 to $O_2 \cdot^-$ during auto-oxidation of sulfonamide hydroxylamine to nitroso, and generation of downstream secondary ROS, such as H_2O_2 (via SOD), and highly active ·OH (in the presence of free transition metal ions, especially Fe(II)); (2) conjugation of highly-electrophilic nitroso metabolite with GSH, which may result in or exacerbate its depletion. Both hypotheses have been verified by experimental results. In an *in-vitro* study of mononuclear leukocytes [21], the toxicity of both hydroxylamine and nitroso of SMX were determined, and the potential protective role of GSH was studied. Findings confirmed that toxicity of SMX hydroxylamine in mononuclear leukocytes (quantified by cell viability values) was attenuated or prevented by exogenous glutathione, which indicated that SMX hydroxylamine conjugation with GSH could be an important detoxicification pathway. Also, in a cell-free experiment, GSH prevented conversion of SMX hydroxylamine to nitroso at the cost of its own oxidation. It was found that, in the initial stage, GSH might nucleophilically attack SMX nitroso for semimercaptal conjugation, which would either be cleaved to for SMX hydroxylamine at a high

concentration of GSH, or undergo isomerization to form more thermodynamically stable (and less active/less dangerous) sulfonamide with a lower concentration of GSH. This might explain theprotective role of GSH in cells in the presence of SMX metabolites. Results for the study of concentration-dependent *in-vitro* toxicity of SMX metabolites, however, showed that significant GSH depletion did not occur until the concentration of SMX metabolite was 3-fold of its LD_{50}. Also, purified GST did not show a significant effect on reactions between SMX-NOH and GSH. Both findings indicated that, although GSH conjugation may not play a major role in detoxification of SMX metabolites, it could still make a significant contribution to sulfonamide-induced oxidative stress.

2.1.3. Isoniazid (Anti-Tuberculosis Agent). Isoniazid (INH) is an organic compound that has long been (and is still currently) used for prophylaxis and treatment of tuberculosis (TB), a contagious disease caused by infection of *Mycobacterium tuberculosis* or other strains of mycobacteria. Although there have always been attempts to screen substitutive medications, INH still serves as the first-line and main drug against TB since its discovery in the early 20^{th} century and clinical use in early 1950s, due to its unique mechanisms of function, high efficiency, and relatively low toxicity.

INH is of a unique and complicated mechanism of therapeutic effect, which was not completely unraveled until decades after its initial clinical application. [22] INH enters mycobacterial cell via passive diffusion as a non-toxic “precursor”. Thereafter it is oxidatively activated by KatG, a mycobacterial cytosolic multifunctional enzyme which mainly serves as catalase/peroxidase but also functions as peroxynitritase and NADH oxidase. Under “normal” conditions, KatG plays an important protective role against the peroxide generated by NAD(P)H oxidase of phagocytes of their hosts upon infection. In

the presence of INH, however, KatG is "hijacked" by INH, which turns it from an antioxidant enzyme into an intracellular "manufacturer" of toxic INH metabolites. This general mechanism is comparable to that of nitrofurantoin (another antibiotic) in the generation of ROS in mammal somatic cells. This will be discussed in later sections. The toxicity of INH decreases in strains of *M. tuberculosis* of the mutant KatG in the narrower heme access channel and, therefore, permits less access for INH to its oxidative site, compared to wild type strains. This verifies the role of the activation of KatG to INH in the mycobacteria. KatG is capable of oxidizing INH in the presence of a number of oxidants, including alkyl hydroperoxides, hydrogen peroxides (possibly a by-product of aerobic metabolism of *M.tuberculosis,* an obligate aerobe), and superoxides (possibly generated by another reaction that could be catalyzed by KatG, i.e., NADH oxidation/O_2 partial reduction).

It was hypothesized that KatG oxidatively activates INH to numerous highly-reactive intermediates, including carbon radicals (such as isonicotinoyl radical), nitrogen radicals (such as isonicotinic hydrazyl radical) or oxygen radicals. This was verified by results of EPR studies. These INH-derived radicals are capable of forming adduct with $NAD(P)^+$ via attacking and covalently conjugating with them at the C-4 position of the pyridine ring of their nicotinamide moiety. The INH-$NAD(P)^+$ adducts are now known to be potent inhibitors of a variety of mycobacterial enzymes, most significantly IhnA, an enoyl-acyl carrier protein reductase, that is involved in synthesis of mycolic acid, one of the main components of cell wall lipids of mycobacteria. Enoyl-acyl carrier protein reductase, which plays a pivotal role in type II fatty acid synthesis, is a common "target" for purpose of anti-bacterial compound synthesis, since the biological processes of fatty

acid synthesis are distinct in prokaryotes and eukaryotes. In case of *M. tuberculosis*, IhnA is inhibited by R-stereoisomer of isonicotinoyl-NAD^+ adduct. Other important mycobacterial enzymes inhibited include Mab A(a NADPH-dependent β-ketoacyl-ACP reductase), *M. tuberculosis* dihydrofolate reductase (which has a different structure than the isozyme in humans), as well as Kas A (another enzyme involved in mycolic acid synthesis).

Besides, these "organic" radicals are also capable of directly attacking biological macromolecules in mycobacteria, in a pattern similar to that of the "inorganic" low-molecular weight radicals, such as $O_2\cdot^-$, NO· and ·OH, which are also generated during KatG activation of INH. Nitric oxide, for instance, has been found to be "scavenged" by mycothiol, which is considered to be an important intracellular antioxidant compound for *M.tuberculosis*. The product, S-nitrosomycothiol, could undergo transnitrosylation and nitrosylate certain proteins. This process is verified to be the underlying reason for dysfunction of several respiratory enzymes in *M. tuberculosis*. Nitric oxide can also undergo an additive reaction with $NAD(P)^+$ to form adducts that inhibit the synthesis of nucleic acid and lipid in mycobacteria. Wild-type strains of M. tuberculosis, especially, are a "knockout" for oxyR, a DNA-binding transcriptional factor that regulates the expression of antioxidant genes (i.e., those coding proteins that mediate peroxide metabolism, redox balance, and peroxide protection, such as the up-take of manganese in response to oxidative stress. This renders them even more vulnerable to an attack by reactive radical species.

The main adverse effects of INH were its potential liver and kidney toxicity (especially with an overdose) and, for subjects of a certain genotype (such as those with

G-6-PDD or other genetic disorders that render them more vulnerable to oxidative stress), hemolytic anemia.

Liver/kidney toxicities are the most common adverse effects of INH, especially in those individuals referred to as "slow acetylators". An intense effort was made in an attempt to unravel the metabolic pathway of INH in the human body, and the main pattern was made clear, as shown in Figure 2.4.

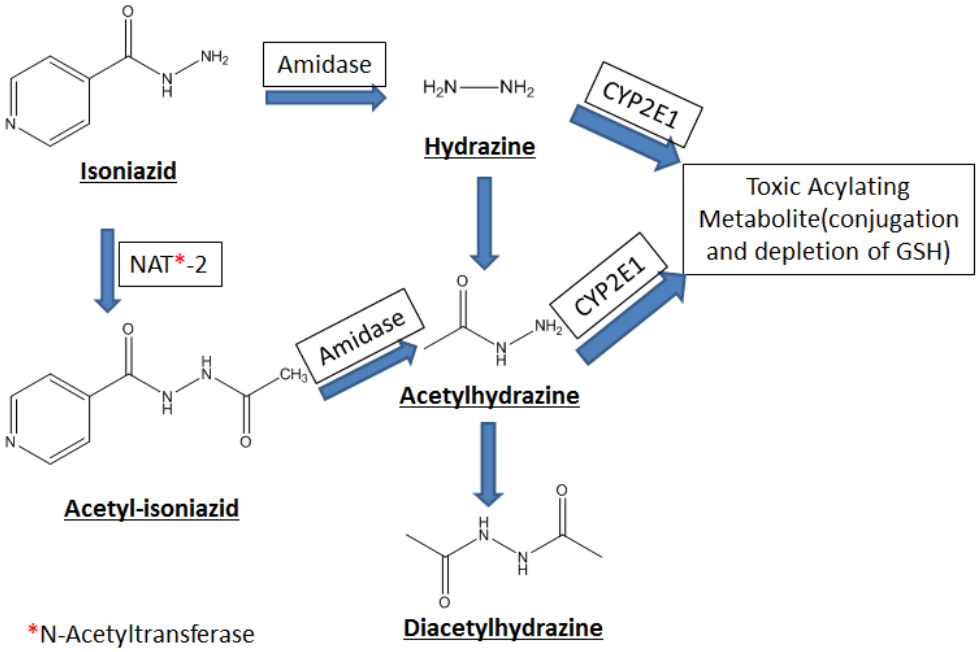

Figure 2.4. Mechanism of INH – Induced Oxidative Stress. As shown in the flowchart, either isoniazid or its N-acetylation metabolite is hydrolyzed, producing hydrazine or acetylhydrazine, which are both metabolized by CYP2E1 to acylating compounds, conjugating and depleting intracellular glutathione.

Upon uptake, INH is mainly acetylated by arylamine N-acetyltransferase (NAT)-2 while using Acetyl-CoA as a co-substrate. Acetyl-INH, therefore, undergoes hydrolysis via catalysis of amidase, yielding isonicotinic acid and acetylhydrazine. The former is normally conjugated with glycine and excreted, whereas the latter is believed to be the key intermediate metabolite that is responsible for INH toxicity. Acetylhydrazine, depending on its own intracellular level as well as activity of the corresponding enzymes, could either be further acetylated by NAT-2 to diacetylhydrazine, a non-toxic metabolite that is ready to be excreted, or activated by CYP2E1 to generate a toxic acylating metabolite, which is capable of conjugating to liver/kidney proteins or depleting GSH, Its toxic effect is exerted in a pattern similar to that of activated metabolite of acetaminophen. [23]

Both *in-vitro* and *in-vivo* studies have been conducted to elucidate the metabolic process and determine the fate of INH. It has been confirmed that conjugation of acetylhydrazine and hepatic protein is subject to mediation of microsomal enzymes. [24] An inhibitor of acyl amidase, the enzyme that hydrolyzes acetyl-INH to isonicotinic acid and acetylhydrazine, alleviates the toxicity of INH, further supporting the hypothesis that INH exerts its toxicity via acetylhydrazine.

Interestingly, the presence of INH, the “mother compound”, will affect the further metabolism of its own intermediate metabolite, which was confirmed by results of a number of experiments, mostly via radioactive isotope labeling. [25][26][27] There are contradictory results for *in-vitro* and *in-vivo* studies, however. It was found that INH inhibits microsomal activation of acetylhydrazine to the toxic acylating agent *in-vitro*; nevertheless, the presence of INH favors this process *in-vivo* and actually diminishes the

physiological reaction of acetylation of acetylhydrazine to diacetylhydrazine. A reasonable explanation would be that INH undergoes acetylation via the same enzymatic system as well, so it would actually compete with acetylhydrazine and "force" it to undergo the other metabolic pathway.

INH causes hemolytic anemia in slow acetylators due to Type II hypersensitivity reaction as well, but the detailed mechanism is different from that of sulfonamide-induced hymolysis. It was found that acetylhydrazine from INH would induce production of a corresponding antibody and bind to it in the blood. The Ag-Ab complex would thereafter be adsorbed on the surfaces of erythrocytes and activate the complementary system. Therefore, the erythrocytes on which the complex "resides" are lysed as "innocent bystanders". On the other hand, the INH-induced hemolysis in G-6-PDD patients has a similar mechanism to that of the sulfonamide-induced hemolysis in them.

2.1.4. Antineoplastic Drugs-Alkylating Agents. "Antineoplastic drug" is a generic term referring to a wide collection of thousands of medicinal drugs that are clinically used for suppression of the growth of an abnormal mass (namely, "a tumor" or "a neoplasm", benign or malignant in nature) in specific tissues and induction of death of the excessively dividing tumor cells. Today, there are a great number of antineoplastic drugs available for optimal therapeutic effects against different types of neoplasms. These drugs could be categorized into several groups, depending on their distinct chemical structures and/or mechanisms of action. The main types are: (1) alkylating agents (the earliest, most "classical" antineoplastic drugs); (2) anthracycline antibiotics; (3) platinum (Pt) – containing antineoplastic drugs; (4) camptothecin; (5) podophyllin derivatives; (6)

Vinca alkaloids; (7) others, such as Bleomycin (BLM), a glycopeptide antibiotic of a unique antineoplastic mechanism.

Among these drugs, camptothecin (topoisomerase I inhibitor), podophyllin derivatives (topoisomerase II inhibitor), and *Vinca* alkaloids (mitosis suppressor due to its ability to bind tubulin dimers) will not be introduced here, since their mechanisms with either therapeutic or adverse effects are barely oxidative-stress related.

"Alkylating antineoplastic drugs", by definition, refer to alkylating agents that are capable of attaching an alkyl group to DNA. Alkylating antineoplastic drugs are mostly strongly electrophilic compounds that can covalently conjugate with negatively-charged DNA by the formation of carbonium ion intermediates or transition complexes with their targets. They exert their therapeutic function mostly by attaching the alkyl group to N-7 on the imidazole ring of guanine (which carries a long pair of electrons and is, therefore, highly nucleophilic) or, occasionally, to O-6 on the pyrimidine ring. Other nucleophilic atoms in the bases of DNA, such as N-1 or N-3 of adenine or N-3 of cytosine, are also prone to be attacked, but to a lesser extent. Generally, such an addition prevents the coiling or uncoiling of DNA, and thereafter approaches normal functioning of the DNA-processing enzyme. "Dialkylating agents", such as cyclophosphamide (CPA) or busulfan, have two or more electrophilic sites in their molecules and, therefore, are capable of attacking more than one N7 of guanine residues. This results in cross-linkage of DNA and makes DNA incapable of strand uncoiling if the two residues are attached to different strands or "limpet attachment" may result if they are affiliated to the same strand. In contrast, "monoalkylating agents" can only react with one guanine moiety. Limpet attachment and monoalkylation, although not capable of preventing DNA helix

from separating, do block the access of DNA-processing enzymes. Consequently, cell division ceases and apoptotic cell death will be triggered if damage to the DNA cannot be repaired. It is worth mentioning, however, that most alkylating antineoplastic drugs are, paradoxically, potential carcinogenic at the same time. It is well known that cell cycles of cells with damaged DNA will be arrested at either the G1/S or G2/M checkpoint to allow for repair of the DNA, and that programmed cell death (apoptosis) will be triggered if the damage is too extensive and attempts to repair it fail, so that the mutation in genes will not be mistakenly inherited by daughter cells. However, if these cells, by any means, overcome these checkpoints without the DNA being properly repaired, and/or if they do not undergo apoptosis due to mutation in certain genes that upregulate anti-apoptotic factors (such as Bcl-2) or downregulate pro-apoptotic factors (such as p53), they will proceed to uncontrolled mitosis and produce an excessive cell mass (neoplasm). This is also accepted as the mechanism of the main adverse effects of alkylating agents and that normal actively-mitotic cells are randomly affected as well. Skin/gastrointestinal tract epithelial cells, hematopoietic cells in bone marrow, and germ cells can be affected, resulting in malabsorption/malnutrition, hair loss, and myelosuppression.

Classical alkylating antineoplastic agents are categorized according to their chemical structure into following groups: Nitrogen mustards (cyclophosphamide, melphalan, chlorambucil, etc), nitroureas (carmustine, steptozocin, etc), and alkyl sulfonates (busulfan).

Regardless of category, a vast majority of alkylating antineoplastic agents are pro-oxidants in vivo, although they have distinct mechanisms. Some (such as carmustine and diethyldithiocarbamic acid) induce oxidative stress by inhibiting certain antioxidant drugs

(this and other antioxidant enzyme inhibitors will be discussed later), while most of the rest are activated by CYP450 and form active intermediate metabolites to conjugate and complete intracellular GSH. Results of experiments in the study of alkylating agent-induced oxidative stress are presented in the following sections.

CPA is categorized into groups of nitrogen mustard alkylating agents. The "nitrogen mustards", which are chemically similar to mustard gas, are typically amines with chlorine substitution on β-carbon. Nitrogen mustards may undergo an intracellular nucleophilic substitution reaction, during which electron-rich nitrogen attacks β-carbon, forming cyclic aminium ion, and chloride detaches as a leaving group. The aminium ion is thereafter attacked by N-7 in guanine and forms a covalent bond (alkylation). To overcome the drawback of the non-selectivity of earlier nitrogen mustards, pharmacists take advantage of the differences in expression by enzymes and modify the structures of drugs accordingly, so that a drug can only be active in the presence or absence of specific enzymes.

CPA, for instance, is one of the selective nitrogen mustards that are highly effective against malignant tumor cells but with minimal toxicity, as compared to the effects of "traditional" chemotherapy medications. The mechanism of CPA toxicity is schemed as in Figure 2.5.

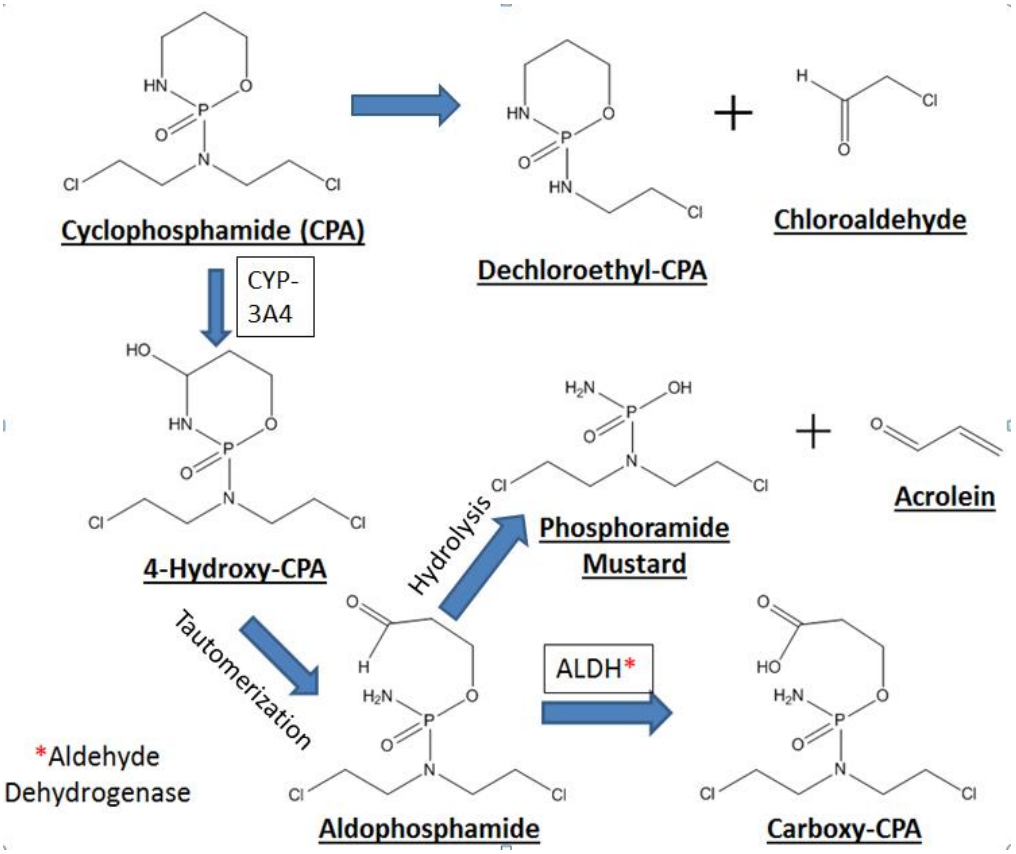

Figure 2.5. CPA Metabolism/Activation. Cyclophosphamide(CPA) is metabolized via following pathways: (1) Oxidation – hydrolysis, producing dechloroethyl-CPA and chloroaldehyde; (2) CYP450-mediated hydroxylation and tautomerization, producing aldophosphamide, which is either oxizdized to non-toxic carboxy-CPA or hydrolyzed to generate DNA-binding phosphoramide mustard, and GSH-conjugating acrolein.

Upon uptake, a small portion of CPA (less than 10%) undergoes an oxidative pathway yielding dechloroethyl-CPA and chloroaldehyde (a neurotoxic agent), whereas a vast majority (over 90%) is oxidatively activated by CYP450 isozymes (mainly CYP3A4, 2B6 and 2C9) to 4-hydroxy-CPA, which swiftly tautomerizes to form aldophosphamide. The latter undergoes either aldehyde dehydrogenase (ALDH) 1A1-mediated oxidation to form inactive, non-toxic carboxy-CPA, or non-enzymatic decomposition to form phosphoramide mustard and acrolein; both are electrophilic and capable of alkylating

DNA. The former, however, remains inactive until its phosphorus-nitrogen bond is cleavaged by intracellular phosphamidase. CPA exhibits its selective toxicity towards malignant cancer cells via two mechanisms: (1) high activity of ALDH (the detoxifying enzyme that oxidizes and deactivates aldophosphamide) in normal cells, which undergo active mitosis and are prone to being attacked by traditional alkylating agents, such as intestinal epithelial cells and hematopoietic cells; (2) high activity of phosphamidase (the activating enzyme that removes phosphoric moiety from phosphoramide mustard, enabling the "mustard" moiety to form cyclic aminium) in a majority of malignant cells. [28] It has been revealed, however, that multiple metabolites of CPA, including chloroaldehyde (the neurotoxic metabolite from side reaction), acrolein and phosphoramide, are capable of forming conjugates with GSH and thereby depleting it, rendering the cells vulnerable to oxidative damage. Other studies [29] indicate that a superoxide anion is generated during the process of decomposition of aldophosphamide to phosphoramide mustard and acrolein. Other oxidases have also been found to be capable of activating CPA to generate GSH-binding (and depleting) intermediate metabolites. The most important enzymes among them are prostaglandin H synthase and horseradish peroxidase [30], or lipoxygenases [31]. Results of experiments to determinate the extent of oxidative stress showed a decrease in the activity of several antioxidant enzymes (SOD, GPx and GR), and depletion of intracellular GSH, as well as lipid peroxidation, upon CPA administration to animals, further confirming the oxidative damage that CPA induces oxidative damage.

2.2. OXIDATIVE STRESS – REDOX CYCLE MECHANISM

"Redox Cycle" is defined as the swift mutual conversion of a substance between its own oxidized and reduced form in inorganic chemistry (such as in transition-metal-containing compounds, where the metal ions alternate between different oxidative states), or interconversion between two related compounds which are considered to be an "oxidized" or "reduced" form of each other in organic chemistry (such as conversion between thiols/disulfide, ketone (or aldehyde)/alcohol, diphenol/quinine, or amine/amine). In biochemistry, alternation between Fe(II)/Fe(III) and Cu(I)/Cu(II) in a variety of iron/copper-centered enzymes are the most common examples of the former, whereas conversion between reduced/oxidized glutathione (or other endogenous thiol antioxidants), NAD(P)H/NAD(P)$^+$ and $FMNH_2$/FMN are prevalent for the latter. In biological systems, redox couples are rarely in equilibrium in physiological conditions (e.g., intracellular GSH constitutes up to 98% of total glutathione under normal conditions). The ratio of reduced/oxidized forms is constantly affected by activities of related enzymes, and is tightly regulated by a number of factors that maintain their proper functioning.

Redox cycle-based enzymatic reactions play pivotal roles in a variety of physiological processes, including generation of ATP through an electron-transporting chain, metabolism, and detoxicification of xenobiotics, as well as signal transduction pathways. It has been noticed, however, that redox cycle reactions of exogenous agents (toxins or medications) in the human body are extremely dangerous. The most common redox-cycling substances that people can be exposed to include bipyridyl herbicides and medicinal drugs bearing biphenol/quinone moieties (or their nitrogen equivalents). These

compounds generally have an “original oxidative form” and exert their toxicity/adverse effects via transformation to highly-active radical intermediates, usually through reducing half-action. The intermediate, in turn, either directly attack and damage nearby biomolecules in the absence of oxygen, or transport the excessive electron to oxygen molecules, generating superoxide anion and converting themselves back to their “original” oxidative state, thereby completing the entire redox cycle. The main types of medicinal drugs that undergo a redox cycle include traditional antimalarial drugs (such as primaquine), anthracycline antineoplastic drugs, and nitrofurans. Bleomycin (BLM), an anti-tumor drug with a unique mechanism of action, induces oxidative stress via its iron center, with a variable valence, and is therefore considered to be a “redox cycle” drug as well. This will be discussed along with anthracycline drugs.

2.2.1. Antimalarial Drugs. Malaria is an infectious disease that is transmitted by female *Anopheles* mosquitoes. It is caused by Plasmodium, a genus of pathogenic protozoans. The vast majority of malaria cases occur when the *P. falciparum*, *P. vivax*, and *P. ovale* in the saliva of an Anopheles mosquito are injected into a human’s bloodstream. After a sexual life cycle, the soprozoites enter the human red blood cells (RBCs) and reproduce, eventually resulting in lysis of the RBC. Malaria can be fatal if not treated properly since infected RBCs might breach the blood brain barrier (BBB), causing life-threatening cerebral malaria. In addition, malaria in pregnant women is a significant cause of stillbirth and infant mortality. Due to the complexity of the life cycle of the *Plasmodium*, various drugs are needed to kill or control this parasite at different stages.

Primaquine (PQ), a member of the 8-aminoquinoline group, is still the only available drug that blocks transmission of Plasmodium since its first-time synthesis decades ago, despite its relatively high toxicity. PQ is versatile in malaria treatment since it not only exerts the effect of a "radical cure" by clearing the latent sporozoites of Plasmodium from the liver after eliminating merozoites from the bloodstream,, thereby preventing a relapse of malaria, but it is also highly cytotoxic towards gametocytes of the pathogen. It interrupts the sexual life cycle of Plasmodium in the mosquito which prevents further transmission of the disease. This is also the primary prophylactic drug recommended for use by travelers to certain regions with high malaria occurrence. The mechanism of PQ killing the parasite (though not fully unraveled) may be by partial interference with the parasite's mitochondrial ETC by its reactive metabolites generated by the CYP450-mediated pathway. This will be discussed further in later sections.

PQ is always associated with severe side effects, despite its great versatility and adaptability and the fact that it is highly effective towards all Plasmodium strains and drug tolerance rarely occurs. The most severe adverse effect of PQ is its hematotoxicity, i.e., the potential to induce hemolytic anemia in people with certain genetic disorders such as glucose-6-phosphate dehydrogenase deficiency (G-6-PDD) or NADH-methemoglobin reductase deficiency. Although not fully determined yet, the mechanism of PQ hematotoxicity has been reported to be associated with its active metabolite, 5-hydroxyprimaquine, which undergoes an intracellular redox cycle and generates secondary free radicals that induce oxidative stress.

Results from experiments conducted by various researchers primarily lead to the agreement that PQ, alone, does not cause "fatal" damage to erythrocytes (especially

normal ones with sufficiently high intracellular GSH). Instead, it is metabolized by a variety of hepatic CYP450 isozymes to different activated intermediates which, in turn, undergo a redox cycle and generate ROS. The entire scheme of this mechanism has been well studied with the major active metabolites being separated, purified, and identified, and their relative toxicity compared to that of PQ.

Several members of the CYP 450 family were found to be responsible for PQ activation. The main ones included CYP2E1, 2B6, 2D6, 3A4, and 1A2. It was found that PQ potently induces transcription of corresponding genes [32]. Among these, CYP2D6 was believed to generate the most reactive PQ metabolites. The administration of a selective inhibitor of CYP2D6 significantly decreased the PQ metabolic rate, whereas (in CYP 2D6-knockout mice) PQ exerted no therapeutic effect, even though the dose was doubled. [33] Several intermediate metabolites were identified, including 5-hydroxylprimaquine (5-HPQ), 5-hydroxyl-6-demethylprimaquine (5-H-6-DPQ), 6-methoxy-8-aminoquinoline (6-M-8AQ), and 5,6-dihydroxyl-8-aminoquinoline (DHAQ). [34] [35] All of these metabolites except 6-M-8AQ could be oxidized intracellularly to form a corresponding quinone-imine (QI). This was confirmed by results of UV-Vis spectrophotometry, IR, NMR, and UPLC-MS, and generated H_2O_2. Spin-trapping EPR failed to detect hemoglobin (Hb) –thiyl or glutathionyl radicals, indicating that these QIs did not directly oxidize GSH nor did they directly react with NADPH. [35] In the presence of ferrodoxin:$NADP^+$ oxidoreductase, however, these QIs underwent a redox cycle to generate H_2O_2 and OH, as well as oxidizing Hb to MetHb. It was confirmed that all of these QIs caused significant generation of MetHb, increased oxygen uptake and erythrocyte osmotic fragility, and depleted non-protein thiol. Among these QIs, that of 5-

HPQ was found to be the most potent. This assumption was supported by the results of a toxicity study of purified 5-HPQ [36]. 6-M-8AQ was prone to oxidation to form corresponding 8-hydroxylamine, which could be further oxidized to nitroso by O_2 to generate $O_2^{\cdot -}$. [37] The main mechanism of PQ toxicity is depicted as in Figure 2.6.

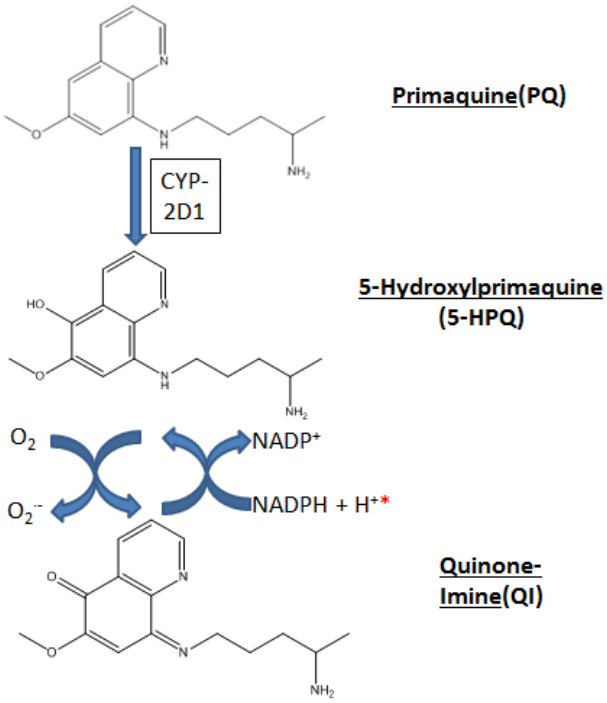

Figure 2.6. Redox-Cycling of PQ Metabolite. Primaquine (PQ) is primarily hydroxylated to 5-hydroxylprimaquine (5-HPQ), which reduces molecular oxygen to superoxide anion. The resulting quinone-imine (QI) can be reduced back to 5-HPQ at the cost of oxidation of NADPH.

2.2.2. Antineoplastic Drugs – Anthracycline Antibiotics; Bleomycin (BLM).

Anthracycline antibiotics and bleomycin exert their antineoplastic effects through distinct mechanisms. It was found, however, that both of them cause oxidative-stress-related adverse effects via redox-cycling mechanisms, which are to be briefly discussed below.

2.2.2.1. Anthracyclines. Anthracyclines are a group of anticancer drugs derived from *Streptomyces peucetius var. caesius*. They are, by far, the most effective antineoplastic compounds and are clinically used for treating various types of malignant tumors related to the reproductive systems, including breast, uterine, and ovary / testicular cancers. They also are used to treat cancers in other tissues/organs, especially those that are resistant to other types of chemotherapy. Most pharmacists believe that the high effectiveness of anthracyclines results from its multiple therapeutic mechanisms. One unique mechanism of action of anthracyclines is its inhibition of topoisomerase II (the intranuclear enzyme cutting of both DNA strands simultaneously and thereby relaxing DNA supercoils) and prevention of DNA transcription or replication. Results of recent studies have shown that anthracyclines do not impede topoisomerase II from combining with DNA. The opposite occurs since it stabilizes the topoisomerase II-DNA complex and prevents the enzyme from dissociating from DNA when necessary. Thereafter, DNA ligase would not be able to repair cleaved DNA, nor would other DNA processing enzymes be capable of approaching and functioning. “Damaged” DNA, in turn, would trigger apoptosis of the cells. There are two main types of topoisomerase II: Top 2α and Top 2β. Top 2α is known to be over-expressed in tumors, but cannot be detected in “quiescent” tissues. Therefore, it is believed to be the basis of anthracycline anti-tumor activity [38]. Other mechanisms of anthracyclines include intercalation

between base pairs of DNA similar to that of an alkylating agent (refer to 1.1.1.4.1. for details) and generation of free radicals that attack and damage various intracellular macromolecules similar to that of bleomycin. It has been hypothesized, however, that these reactive species are possibly the culprit that causes the adverse effects of anthracyclines, the most common and life-threatening of which is cardiotoxicity. In the following section, doxorubicin (DOX, previously known as adriamycin) will be discussed as representative of anthracycline mechanisms and oxidative stress-related adverse effects.

Despite its high effectiveness in treating leukemia, lymphoma, and breast cancer, doxorubicin (DOX) has significant cardiotoxicity, which limits its clinical application. It has been revealed that DOX may trigger apoptosis or cause necrosis of cardiomyocytes with low or high dose, respectively. Comprehensive studies have been conducted of DOX cardiotoxicity, which was found to be mainly due to the excessive ROS it generates from the enzymatic redox-cycle. DOX redox-cycling reactions are schemed in Figure 2.7.

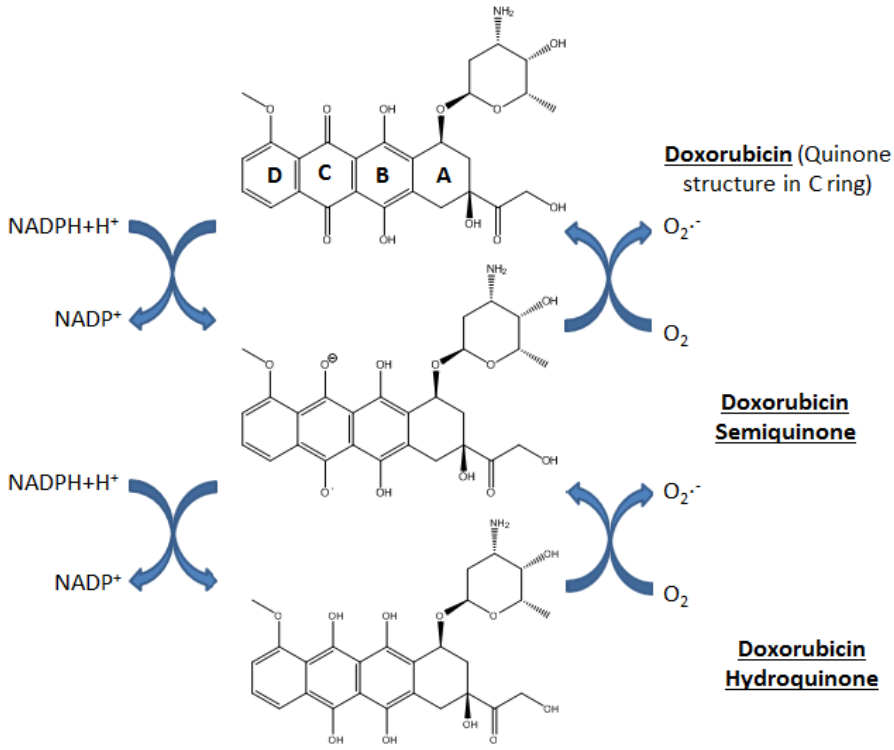

Figure 2.7. Structure and Redox Cycle of DOX. Doxorubicin induces oxidative stress via several pathways. The major one involves redox cycling on its quinone moiety of its C ring. One equivalent of doxorubicin could deplete 2 equivalents of NADPH and generate 2 equivalents of $O_2 \cdot^-$.

The cardiomycytes are prone to oxidative damage due to their high content of mitochondria which, under certain types of stress, might turn into a major site of intracellular ROS generation. In case of DOX-induced cardiotoxicity, it is mainly the quinone moiety (C ring) of DOX that undergoes a one-electron reduction to form semiquinone (DOX-SQ) which, in turn, reduces O_2 to generate $O_2 \cdot^-$. Several enzymes were found to be capable of catalyzing the conversion of DOX to DOX-SQ, including NADPH-CYP450 reductase [39], glutathione reductase [40], as well as a variety of other oxidoreductases. The one that is most commonly involved in DOX reduction, however, is

NADH dehydrogenase (which is also known as Complex I of electron-transporting chain, ETC, on the inner membrane of mitochondrion) [41][42][43]. In mitochondrial ETC, DOX-SQ could be oxidized by cytochrome c which, in turn, "leaks" electrons to O_2. [44] The superoxide anion produced, in turn, will be further metabolized to H_2O_2 by SOD and generate OH in the presence of free iron. The other possible pathway of OH generation from $O_2\cdot^-$ is that $O_2\cdot^-$ could donate its electron to ATP-chelated ferric ion, reducing it to ferrous ion which catalyzes a Fenton-like reaction [42]. Iron plays a very important role in DOX-induced oxidative stress in cardiomyocytes. Physiologically, intracellular iron concentration is tightly regulated by a series of iron-binding and transporting proteins. DOX, however, has been found to be capable of disrupting iron balance via multiple pathways. In mitochondria, the main source of free iron, upon DOX-administration, is the 4Fe-4S center of aconitase, which is vulnerable to oxidative stress and could release Fe(II) upon attack by $O_2\cdot^-$ [45]. The iron-free aconitase has been found to behave like an iron-regulatory protein (IRP) that upregulates transferrin expression (plasma iron-binding protein mediating an iron import via a transferrin receptor on the cell membrane) and downregulates ferritin expression ("safe" intracellular iron-storing protein) [44]. The two derivatives of DOX, DOX-ol (product of A ring-α carbonyl reduction via NADPH dependent carbonyl reductase) and DOX-Fe(III) complex (ferric ion "bridges" between oxygen atoms of B and C rings) would dysregulate expression of IRP-1 as well, and further exacerbate disruption of the iron balance. Binding of DOX or its metabolites to cardiolipin (a lipid in cardiac mitochondria that keeps the inner membrane intact and maintains proper ETC function) will release caspase-3 directly, or via the p38-mediated process, triggering apoptosis. [42][45]

2.2.2.2. Bleomycin (BLM). Bleomycin (BLM), a glycopeptide antibiotic that is produced by *Streptomyces verticillus.*, refers to a group of structurally-related chemotherapeutic agents used primarily in the treatment of lymphomas, squamous cell carcinomas, testicular tumors, and malignant pleural effusions. The antineoplastic effect of BLM is believed to be due to the formation of a BLM–oxygen complex that binds to DNA and cleaves to the phosphodiester– deoxyribose backbone. The most common and severe (essentially fatal) adverse effect of BLM is pulmonary fibrosis (PF), the accumulative and irreversible replacement of normal lung parenchyma with excessive connective tissues and, therefore, loss of lung elasticity, poor ventilation, and oxygenation in patients. [46] PF is the only dose- and time- dependent adverse effect of BLM and is usually the limiting factor of BLM administration.

The mechanism of action of BLM has been comprehensively studied, and essential details determined, as shown in Figure 2.8.

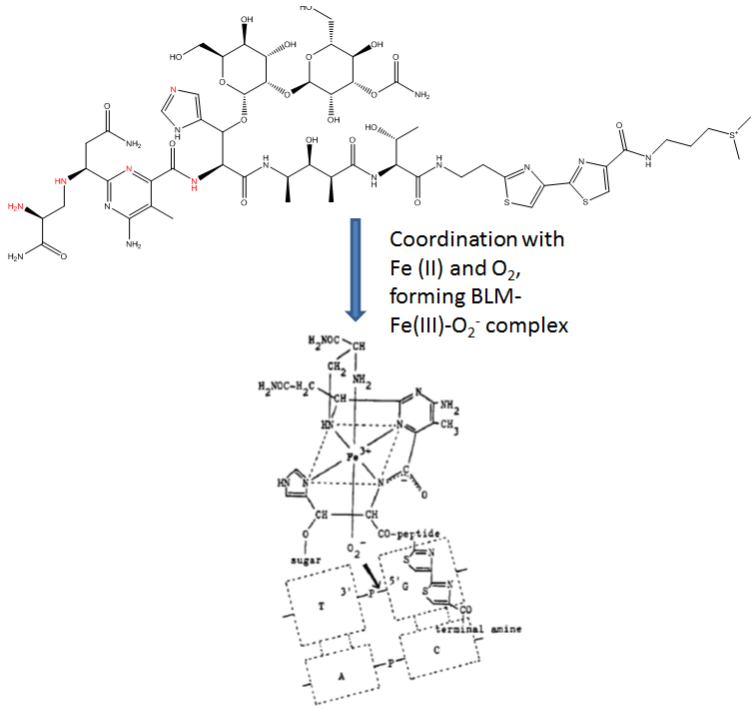

Figure 2.8. Structure of BLM and Its Complex with Fe(II) and O_2. As shown in the figure, the Fe(II)-binding moiety of bleomycin A2 forms coordination bonds with Fe(II). The five nitrogen atoms (red) occupy all planar and one axial position. The resulting complex thereby combines with DNA, alters its configuration, which favors O_2 occupation of the other axial position. [47]

The results of several *in-vitro* studies revealed that BLM molecules could anaerobically bind either ferrous or ferric ions. The BLM-Fe(II) complex could simultaneously combine with DNA, which resulted in a change in its configuration, making it easier for O_2 molecules to bind, followed by "automatic" oxidation and breakage of DNA strands. [47] NMR results indicated that ferrous ion had formed five coordinations respectively with primary and secondary amine of b-aminoalanine, N-5 of pyrimidine ring, secondary amide attached to C4 of the pyrimidine ring, as well as N-1 of b-histidine ring, resulting in a ternary complex that allowed O_2 to be the sixth ligand for

ferrous ion coordination [48]. The O_2-binding spectra of the BLM-Fe(II) complex was similar to that of heme-containing oxygenase, while carbon monoxide or cyanide could inhibit O_2 binding to both. Superoxide anion and hydroxyl radicals were found to have been generated, the "non-free radical", i.e., BLM-Fe(III)-O_2^{2-}· also existed. All of these radicals were interacted with DNA, which caused breakage of the DNA strand and released the free base, especially in G-C-G-T sequences). The results of UV-Vis spectrophotometry and spin-trapping of the BLM-Fe(III) complex, by contrast, showed it was not capable of breaking DNA unless a reducing agent was present. [47] In the presence of a reducing agent (such as $Na_2S_2O_4$, $NaBH_4$, or L-ascorbic acid), however, BLM-Fe(III) was prone to reduction. Some enzymes, such as NADPH-cytP450 reductase or NADH-cyt b5 reductase, could also facilitate the redox cycle of BLM-Fe(III) and generate ROS, resulting in DNA breakage, lipid peroxidation, GSH depletion, and other oxidative damages. [49][50] Lungs were shown to be vulnerable to BLM-induced oxidative stress, since it lacked BLM hydrolase, the protective enzyme that deaminizes BLM and attenuates its ability in O_2 binding and ROS generation[48]; as well as the fact that lungs are the site of gas exchange and exposure to atmospheric O_2.

2.2.3. Antibiotics – Nitrofurans. Nitrofurantoin (NFT), a synthetic nitroaromatic compound belonging to the nitrofuran group, has been used as an antibiotic drug since 1952 and is still widely prescribed today. It has proved to be highly effective against various pathogenic bacteria that cause urinary tract infections, especially those Gram-negative and/or β-lactamase-producing species that are insensitive to penicillin and its derivatives, such as *E.coli*, *Staph. Saprophyticus*, and *Enterococci*.

Although complicated, the NFT mechanism hypothesis states that, upon uptake, NFT enters bacterial cells and is reduced to its active form by the cytoplasmic enzymes in its nitro group, and that, thereafter, either an attack or a break with the nearby circular DNA reduces O_2 to $O_2\cdot^-$. Further investigation is needed. Similar redox cycling occurs in mammalian cells as well. In prokaryotes, this step is generally catalyzed by nitrofuran reductase (NADPH nitroreductase), while in eukaryotes, it could be NADPH-cytochrome P450 reductase (in ER), xanthine oxidase, ETC enzymes (such as NADH-ubiquinone oxidoreductase and cytochrome c reductase), or even glutathione reductase [51]. NFT redox cycling process is depicted in Figure 2.9.

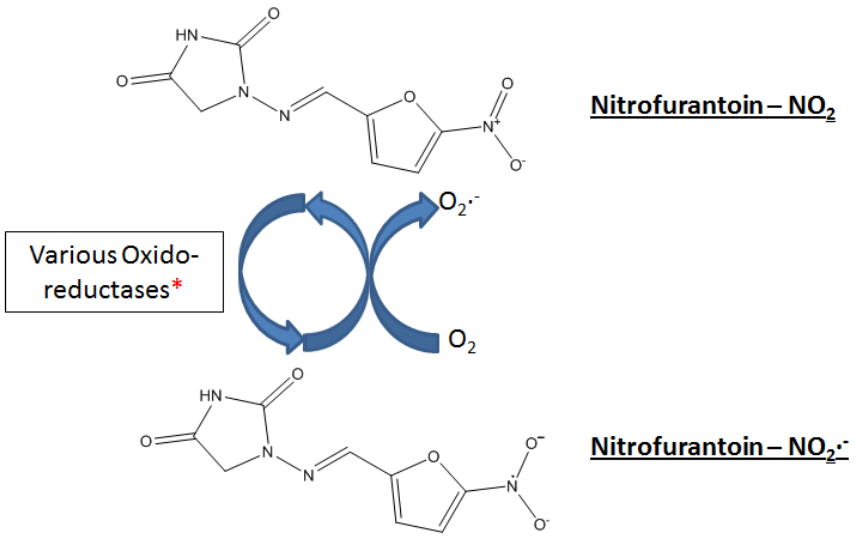

Figure 2.9. Redox Cycle of NFT. Nitrofurantoin is reduced at its nitroso group by different enzymes in prokaryotes and eukaryotes, followed by re-oxidation and generation of $O_2\cdot^-$. The redox-cycling reaction has a higher rate in prokaryotes. Therefore, nitrofurantoin is more toxic to the pathogenic bacteria than to host human.

It was found that diphenyleneiodonium chloride, a known NADPH oxidase and other flavoenzyme inhibitor, is capable of impeding the process of NFT redox cycling [52]. Recent studies state that, in eukaryotes, NFT is suspected of being pro-oxidant by attacking key enzymes in the mitochondrion and causing its dysfunction, as well as damaging enzymes containing iron-sulfur clusters and mobilizing iron ions. It also expanded the labile iron pool, which serves as a catalyst of the Fenton reaction.

It was found that bacteria reduced the nitro group more rapidly than humans, which minimized oxidative damage to the host. However, NFT was contraindicated in patients with glucose-6-phosphate dehydrogenase deficiency due to the risk of fatal hemolytic anemia. In addition, it may cause pulmonary fibrosis, polyneuritis, and drug-induced autoimmune hepatitis, especially in the elderly patients.

2.3. OXIDATIVE STRESS-ANTIOXIDANT ENZYME INHIBITING MECHANISM

Not many types of medicinal drugs induce oxidative stress by the mere inhibition of antioxidant enzymes (CAT, SOD, GPx, GR, GST, etc.). The enzyme-inhibiting drugs are mainly antineoplastic medications, especially alkylating agents and sulfoximines. Here is discussed the mechanism of action of buthionine sulfoximine (BSO), which is clinically used to accompany administration of alkylating agents to potentiate their cytotoxicity in tumors. [53] [54]

BSO is an irreversible inhibitor of γ-glutamylcysteine synthetase (GCS), the first and rate-limiting step of GSH de novo synthesis. In the presence of Mg(II)-ATP, γ-GCS phosphorylates L-glutamate (Glu) forms γ-glutamyl phosphate, which binds to the

enzyme. The intermediate further undergoes condensation with L-Cysteine (Cys) to form γ-glutamylcysteine. BSO, as a structuralanalogue of γ-glutamylcysteine, reacts with Mg(II)-ATP to form BSO-phosphate, which combines tightly, but not covalently, to active sites of γ-GCS, by fitting its sulfoximine in the site normally for γ-carboxylic of glutamate, and the S-butyl group in the site normally for a Cys combination. Therefore, the reaction cycle cannot be finished and no more γ-glutamylcysteine can be generated to further produce GSH. By depleting intracellular GSH in tumor cells, BSO significantly increases their vulnerability to other antineoplastic drugs with pro-oxidative effects. The synergistic cytotoxicity provides the strategy for treatment of drug-resistant tumors. [55]

2.4. POTENTIAL PROTECTIVE ROLES OF ANTIOXIDANT AGAINST MEDICINAL DRUG-INDUCED OXIDATIVE STRESS

In general, it seems reasonable to hypothesize that antioxidants would help to alleviate the oxidative stress-related adverse effects of these medicinal drugs. Clinically, however, there are more concerns regarding the safety/toxicity of the antioxidants themselves, their possible interactions with the main drugs administrated, and whether they would attenuate the therapeutic effects of the main drugs, especially those that exert therapeutic and toxic effects with the same or similar mechanisms. Comprehensive studies have been made of the possible roles of antioxidants in preventing oxidative damage induced by medicinal drugs. It has generally been found that antioxidants do play a positive role to some extent.

The biological antioxidants studied fall into two main categories: (1) Low molecular-weight compounds that are chemical reducing agents and are “sacrificed” upon

exertion of their protective roles (i.e., at the cost of their own oxidation, such as ascorbic acid, tocopherol, reduced glutathione, and their derivatives/precursors, including N-acetylcysteine and N-acetylcysteineamide, the main antioxidant drugs tested in this laboratory); and (2) relatively high molecular-weight compounds/complexes, usually polyunsaturated macrocyclic aromatics that form a coordination bond with transition metal ions, referred to as "catalytic antioxidant mimetics" because they mimic the structures/functions of endogenous antioxidant enzymes (CAT, SOD, GPx, GR, GST). They rapidly react with and diminish ROS, and are themselves regenerated thereafter. Also, there are other compounds that may exert intracellular "antioxidant" effects although they are not chemically reductive agents. These compounds may either chelate free transition metal ions to prevent them from catalyzing hydroxyl radical-generating Fenton reactions, or inhibit certain enzymes that participate in generation of active metabolites of some medicinal drugs (mostly CYP450). A variety of plant extracts, which are essentially a natural antioxidant solution (mostly a mixture of polyphenols, flavonoids, and thiols) have also been studied to determine their protective effectiveness.

2.4.1. The Protective Role of N-Acetylcysteine (NAC). Reduced glutathione (GSH) is the main endogenous antioxidant that defends against oxidative stress. It serves as a substrate for (1) a conjugation reaction, catalyzed by glutathione S-transferase, which plays a pivotal role in phase II of drug metabolism; (2) a reduction reaction, catalyzed by glutathione peroxidase, which is essential for detoxicification of peroxide radicals. Accordingly, the repletion of the intracellular GSH pool is presumed to be beneficial in cases of oxidative damage. However, direct glutathione administration is unlikely to significantly increase the circulatory GSH level due to the hydrolysis of GSH by

intestinal and hepatic γ-glutamyltransferase [56]. Therefore, administration of metabolic precursors of GSH synthesis is considered to be a possible strategy for increasing GSH levels.

N-acetylcysteine (NAC) is a synthesized thiol and derivative of L-cysteine (Cys), an endogenous amino acid and the precursor of de novo synthesis of GSH. Upon administration, NAC is either deacetylated extracellularly to generate L-cysteine, which is transported into cells via an alanine-serine-cysteine transport system, or diffused into the cell to undergo hydrolysis via acylase. The L-cysteine produced from NAC could participate in the synthesis of γ-glutamylcysteine, which is the first and rate-limiting step of GSH synthesis. Therefore, NAC serves as an indirect precursor of GSH. [57] Besides, NAC is also known to have direct ROS-scavenging power due to its reducing sulfhydryl group [58] [59][60] and the chelating capability of heavy metal ions (including iron and copper). These are comparable to metallothionein, the endogenous cysteine-rich heavy metal chelator [61] that is known to be the catalyst of the Fenton reaction that generates highly active ·OH. NAC is also found to have anti-inflammatory and anti-apoptosis capability by inhibiting the nuclear factor (NF)-κB (the pro-inflammatory transcription factor) and preventing activation of the p38 MAP kinase (the upstream kinase that mediates the signal transduction pathway that leads to apoptosis). [58] The protective role of NAC against oxidative stress induced by medicinal drugs has been studied extensively. It was found that NAC, to a varying extent, showed its effect in alleviating oxidative damage resulting from drug administration, regardless of the mechanism of oxidative stress. In fact, NAC (because of its GSH-boosting capability) has been approved for both oral and intravenous administration as an antidote for an acetaminophen overdose that

induces CYP450-mediated oxidative stress. [63] [64] It has also been proved to be effective in alleviating oxidative stress induced by medicinal drugs that exert their pro-oxidative effects via different mechanisms. These include CYP-450-mediated oxidative drugs such as sulfonamides [65][66], isoniazid[63] and busulfan[64], redox-cycling drugs such as primaquine [69] and doxorubicin [70], as well as antioxidant enzyme inhibitors such as buthionine sulfoximine [71].

The main concern about NAC, however, is its bioavailability. NAC is a derivative of amino acid, and its α-carboxylic group, which is negatively charged at physiological pH, prevents it from freely diffusing across the hydrophobic cell membrane. Therefore, modification of NAC molecule is needed. N-acetylcysteineamide (NACA), the amide form of NAC, is hypothesized to be of higher lipophilicity and higher bioavailability, thereby requiring a lower dose and a shorter dosing time. [56]

2.4.2. Antioxidant Potential of *Sutherlandia frutescens*. *S. frutescens* (SF) is a legume native to southern Africa (the Cape of Good Hope and nearby regions), where it is one of the best-known and most well-respected general medicinal plant in the native habitant of the Cape [72]. It has been found to have great versatility in its effects on various types of disorders, including infections (such as flu, TB, chicken pox, urinary tract infection, and diarrhea) [73]; cancer [74] and inflammatory diseases [75] [76]; and HIV/AIDS [77] [78]. It might also be helpful in treating Type II diabetes mellitus or other autoimmune diseases [79]. Though not a magic cure for all of the diseases above, *S. frutescens*, in some ex-/in-vivo studies, does show potency in alleviating symptoms or delaying the progression of these disorders and, therefore, has drawn the interest of medical researchers.

SF was of interest because of its possible protective role against medicinal drug-induced oxidative stress. The present study, therefore, was conducted as a preliminary study to confirm its antioxidant potency.

The anti-inflammatory effect of SF (at least partially) relies on its capability of scavenging phagocyte-derived oxidative species [76]. It was found that an extract of SF scavenges free radicals in both the cell-free system (which was verified by the results of a DPPH assay) as well as *in-vitro* (as for phagocyte-derived reactive oxygen species). The overall antioxidant capability of SF, however, has not been thoroughly studied. Phytochemical investigations of the SF plant showed that it contains significant amounts of γ-amino butyric acid and L-canavanine, pinitol, flavonol glycosides, and triterpenoid saponins, that may be pharmacologically relevant [72]. The great diversity in the possible antioxidant metabolites present in SF indicates that SF extract might be a promising candidate as antioxidant supplementation, as well as a solution for medicinal-drug-induced oxidative stress.

3. *IN-VITRO* STUDIES TO COMPARE PROTECTION OF N-ACETYLCYSTEINE AND N-ACETYLCYSTEINEAMIDE AGAINST STRESS INDUCED BY OXIDATIVE MEDICINAL DRUGS

N-acetylcysteine (NAC), a synthetic derivative of L-Cys, is a well-known thiol-containing antioxidant that has a clinical history that spans more than 50 years. [80] It is mainly used as an antidote for acetaminophen overdose [81], a mucolytic agent [82] and a nutritional supplement. [83] NAC exerts its antioxidant role mainly via the following mechanisms: (1) It enters the cell and undergoes hydrolysis (deacetylation) to yield L-Cys, the precursor of de-novo synthesis of GSH; (2) It scavenges free radicals and chelates heavy metal ions, including transition metal ions that could catalyze ·OH - generating Fenton reaction. In addition, it has also been found to have anti-inflammatory and anti-apoptotic effects that mediate signaling-transduction pathways by reducing the sulfhydryl group of redox-sensitive factors. NAC was proved to be protective against oxidative stress induced by a large number of pro-oxidative medicinal drugs and other compounds, regardless of their mechanisms. N-acetylcysteineamide (NACA), the amide form of NAC, was designed and synthesized as a substitute for NAC because of its higher lipophilicity and bioavailability, as well as its ability to cross the blood-brain barrier [84]. With the positive results obtained with regard to the protective role of NACA when administering some medicinal drugs [85], it is reasonable to hypothesize that NACA would be a promising antioxidant to protect against oxidative damage induced by pro-oxidative medicinal drugs.

Two different studies were conducted to determine the *in-vitro* protective effect of NACA against the following two medicinal drugs with distinct mechanisms of action: Bleomycin (BLM) and Nitrofurantoin (NFT). NAC was also included as a comparison.

The NFT experiments were performed with human hepatoma (HepaRG) cells, whereas those of BLM were performed with human alveolar basal epithelial (A549) cells. Details of these experiments are included in the "Materials and Methods" section.

3.1. EXPERIMENTAL DESIGN

In general, treatment of cells with pro-oxidants (regardless of their mechanisms of action) results in time-and-dosage-dependent oxidative damage and cell death, increase in intracellular reactive oxygen species (ROS) levels and the extent of lipid peroxidation, decrease in intracellular GSH levels and the GSH/GSSG ratio, as well as alteration of activities of several antioxidant enzymes, in responses to oxidative stress. These "oxidative parameters" of cells will be measured after giving doses of the oxidative medicinal drugs, with or without pre-administration of NAC/NACA.

Besides the "common" oxidative damages, however, certain oxidative drugs might also disrupt specific organelles or cause dysfunction/death of cells in some unique way, depending on the mechanism of the induction of oxidative stress. Cell models derived from different origins might also response differently due to the discrepancy in expression in some proteins (especially antioxidant enzymes or sensitive redox "indicators"). The specificities of the drugs and/or cell lines are to be discussed in the following sections.

BLM is the collective name for a group of structurally-related glycopeptide antibiotics used as antineoplastic drugs clinically. It is only time-and-dose-dependent with a fatal adverse effect of pulmonary fibrosis (PF), which has an intricate mechanism and pathogenesis. It has been implied that oxidative stress is an important factor in BLM-

induced PF. BLM chelates intracellular ferrous ion, forming a complex which might undergo a redox cycle and generate oxygen free radicals. Immobilized human alveolar basal epithelial cells (A549) were used as an *in-vitro* model to study (1) the oxidative effects of BLM on A549, and (2) to determine whether NACA provides any protection against BLM-induced cytotoxicity. NAC was used as a comparison in some parts of the experiments.

NFT, a member of nitrofuran antibiotics, is used clinically for treatment of urinary tract infection or other infections caused by Gram-negative bacteria, which are recalcitrant to b-lactam antibiotics (such as penicillin). NFT exerts its antibacterial effect via reduction of its nitro group by prokaryotic cytosolic enzymes and consequently, re-oxidation by molecular O2 and generation of O2·- that cleaves bacterial circular DNA. Such a redox cycle also occurs in eukaryotic cells, but is believed to be of a much lower rate. NFT could, however, result in life-threatening hemolysis in patients with G-6-PDD, or related genetic disorders, as well as cause PF and hepatic necrosis upon overdose or prolonged administration. NFT adverse effects are believed to involve oxidative stress of the mechanism discussed above. It has been indicated that NFT reversibly inhibits GR as well as several oxidoreductases in mitochondria, probably due to the fact that NFT might serve as a "substrate" of these enzymes and, therefore, disturb their normal functions. In this study, the HepaRG cell line was used to study the cytotoxicity of NFT and the protective role of NACA. NAC was used in part of the experiment.

3.1.1. Cytotoxicity Studies. The Calcein AM assay was used in cytotoxicity studies. Calcein AM is a dye that is structurally related to phenolphthalein. It could be used to determine the cell viability of most eukaryotic cells. In living cells, non-

fluorescent calcein AM is converted to green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. The intensity of the fluorescence is proportionally indicative of the number of living cells. The fluorescence could be measured with an excitation wavelength of 485nm and an emission wavelength of 530nm.

3.1.1.1. Studies of dose-dependent cytotoxicity of the oxidative drugs. The purpose of this study is to assess the toxic effect of different doses of specific medicinal drugs on each cell line. The target is to determine the dose of each drug, which corresponds to 60-70% of cell viability that would be optimal for evaluation of NAC/NACA protective roles.

The cells were seeded in a 96-well plate at a density of approximately 1×10^4 cells/well, and allowed to grow in complete medium at 37°C for 24 hours. Thereafter, the complete medium was replaced by a serum-free medium (SFM, vehicle) or appropriate drugs of various concentrations that had been dissolved in SFM for 24 hours. The cells were then washed three times and incubated with 100μM of calcein AM for 30 minutes, followed by measurement of the fluorescence. The optimal doses of tested drugs were determined according to a dose-response curve.

3.1.1.2. Cytotoxicity of NAC/NACA. The purpose of this study is to assess the toxicities of different concentrations of NAC/NACA to cells, possible toxic effect of NACA, the more important antioxidant. Considering the short time required for pre-treatment of antioxidants, the highest non-toxic dose of NACA was chosen to maximize the possible protective effect. For further experiments, NAC (if used) would be at the same concentration as NACA so that the results would be comparable.

3.1.1.3. NAC/NACA protection on oxidative-drug-treated cells. The purpose of this study is to roughly assess the protective effects of different NAC/NACA doses on the cells treated with specific oxidative drugs.

The cells were seeded and incubated, as described in 3.1.1.1. Thereafter, they were pre-treated with different concentrations of NAC or NACA dissolved in SFM for 2 hours, followed by treatment with specific drugs for 24 hours.

3.1.2. Measurement of Intracellular Reactive Oxygen Species (ROS). Administration of oxidative drugs, regardless of their mechanism, would result in generation of intracellular ROS, another marker of the severity of oxidative stress. Intracellular ROS could be measured using 2′, 7′-Dichlorofluorescin diacetate (DCFH-DA), which is a cell-permeable, non-fluorescent dye. [8] DCFH-DA is de-esterified intracellularly (DCFH) and is converted to fluorescent 2′,7′-dichlorofluorescein (DCF)upon oxidation. Therefore, the intensity of the fluorescence is proportionally related to the quantity of intracellular ROS. The fluorescence could be measured with an excitation wavelength of 485nm and an emission wavelength of 520nm.

3.1.2.1. Study of ROS generation upon treatment of oxidative drugs. The purpose of this study is to study the effect of different doses of specific oxidative drugs on ROS generation. The cells were seeded, as described in 3.1.1.1, washed and incubated with 50μM DCFH-DA in SFM for 30 minutes, followed by treatment with different concentrations of specific oxidative drugs of different concentration for 1hour and measurement of the fluorescence.

3.1.2.2. Study of ROS-scavenging potency of NAC/NACA. The purpose of this study is to assess the dose-dependent radical scavenging power of NAC/NACA.

The cells were seeded, as described in 3.1.1.1, washed and pre-treated with different concentrations of NAC/NACA in SFM for 2 hrs, followed by the loading of DCFH-DA dye. The cells were then treated with specific oxidative drugs at concentrations corresponding to 60-70% cell viability and the fluorescence was measured afterwards.

3.1.3. Oxidative Stress Studies. As discussed above, oxidative stress decreases the intracellular GSH level and the GSH/GSSG ratio, and increases the level of MDA (the product of lipid peroxidation), as well as altering the activities of major antioxidant enzymes such as CAT, GPx, GR, SOD, and GST. These parameters are, therefore, useful markers that indicate the extent of oxidative stress. In these studies, the cells were treated with specific oxidative drugs with or without the pre-treatment of NAC/NACA, and measured the intracellular levels of these markers, in an effort to elucidate the protective roles of NAC/NACA against oxidative stress induced by these medicinal drugs.

The cells were seeded at an initial density of 5×10^6 per flask ($25cm^2$) with 5mL of complete medium. After cells attached, the culture flasks were divided into six groups with quadruplicates within each group, unless otherwise mentioned. All agents were dissolved in SFM.

1) Control (n=4): 2 hours of SFM, followed by 24 hours of fresh SFM;
2) Oxidative drug only (n=4): 2 hours of SFM followed by 24 hours of specific oxidative drug;
3) NAC only(n=4): 2 hours of NAC, followed by 24 hours of SFM;
4) NACA only(n=4): 2 hours of NACA, followed by 24 hours of SFM;
5) Oxidative drug + NAC (n=4): 2 hours of NAC, followed by 24 hours of specific oxidative drug;

6) Oxidative drug + NACA (n=4): 2 hours of NACA, followed by 24 hours of specific oxidative drug.

After treatment, the cells were trypsinized (unless otherwise mentioned) and collected by centrifugation. The following oxidative stress parameters were determined:

(1) Levels of reduced glutathione, which is the main intracellular thiol for defense of oxidative damage;
(2) Levels of oxidized glutathione;
(3) Levels of malondialdehyde (MDA), the marker for lipid peroxidation;
(4) Activities of antioxidant enzymes: catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD).
(5) Mitochondrial membrane potential, the loss of which could be either a result or a cause of oxidative stress, depending on the mechanism of the oxidative drug. It will be discussed in detail in later sections.

3.2. MATERIALS AND METHODS

3.2.1. Materials. The human alveolar basal epithelial cells (A549) and human hepatoma cells (HepaRG) were obtained from Invitrogen. NACA was gifted by Dr. Glenn Goldstein (David Pharmaceuticals, New York, NY). N-(1-pyrenyl)-maleimide (NPM) was obtained from Sigma-Aldrich (St. Louis, MO). High performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were bought from Sigma-Aldrich (St. Louis, MO).

3.2.2. Cell Culture. Both cell lines used were adhesive cell lines, and techniques of cell culture are similar.

3.2.2.1. Culture of human alveolar basal epithelial cells (A549). The human lung carcinoma pulmonary type II-like epithelium cells (A549) were seeded in 25 cm^2 tissue culture flasks coated with type 1 rat tail collagen (Sigma-Aldrich, St. Louis, MO) and maintained in F-12 Ham's medium with 10% heat-inactivated fetal bovine serum in humidified 5% CO_2-95% air at 37°C. The culture medium was changed every 3 days.

3.2.2.2. Culture of human hepatoma cells (HepaRG). The human hepatoma cells (HepaRG) were seeded in 75 cm^2 tissue culture flasks coated with type 1 rat tail collagen (Sigma-Aldrich, St. Louis, MO). They were maintained in William's E medium supplemented with 10% FCS, 100U penicillin, 100 ug/ml streptomycin, 5 ug/ml insulin, and hydrocortisone in humidified 5% CO_2/95% air at 37 °C. The culture medium was renewed every 3 days. After about 2 weeks, when the flask was full, the cells were shifted to the same medium supplemented with 2% DMSO (differentiation medium). The medium was renewed every 2 to 3 days for 2 more weeks. After that, the medium was switched to a DMSO-free medium for 1 day, and the cells were ready for experiments.

3.2.3. Preparation of Cell Homogenate. After treatment, the cells were washed twice with PBS (pH=7.4), trypsinized and collected by centrifugation at 4°C, 3500RPM for 10min. The cell pellets collected were homogenized on ice with SBB (pH = 7.5), unless otherwise mentioned. The homogenate was then centrifuged at 4°C, 3500RPM for 10min to remove debris. The clear supernatant was used to assess levels of reduced/oxidized glutathione, MDA, and activities of antioxidant enzymes.

3.2.4. HPLC System. The HPLC system (Thermo Electron Corporation) consisted of a Finnigan TM Spectra SYSTEM SCM1000 Vacuum Membrane Degasser, a Finnigan TM SpectraSYSTEM P2000 Gradient Pump, a Finnigan TM SpectraSYSTEM AS3000 Autosampler, and a FinniganTM SpectraSYSTEM FL3000 Fluorescence Detector (λ_{ex}=330 nm and λ_{em}=376 nm). The HPLC column was a Reliasil ODS-1 C_{18} column (Column Engineering, Ontario, CA, USA).

3.2.5. Fluorescence Plate Reader. A FLUOstar OPTIMA microplate reader (BMG Labtechnologies. Inc, Durham, NC) was used for measuring fluorescence in the experiments for cell viability and for intracellular ROS studies.

3.2.6. Fluorescent Microscope. The fluorescent microscope used for measuring mitochondrial membrane potential (Section 2.2.15) was an Olympus IX51 inverted microscope at 400× total magnification with a UPLFLN 60× NA 1.25 objective. FITC (ex 482/35, 506DM, em 536/40) and Texas red (ex 562/40, 593DM, em692/40) filters were used (Brightline). Images were captured with a Hamamatsu ORCA285 CCD camera. The shutters, filters, and camera were controlled using Slide Book software (Intelligent Imaging Innovations, Denver, CO, USA).

3.2.7. Spectrophotometric System. The spectrophotometric instrument used for determination of activities of antioxidant enzymes was a Hitachi U-2000 double-beam UV-Vis spectrophotometer.

3.2.8. Calcein AM Assay. As discussed in Section 2.1.1, a Calcein AM Cell Viability Assay Kit (Biotium, Inc. Hayward, CA, USA) was used in cytotoxicity studies. This assay used calcein AM (CAS name: Glycine, N,N'-[[3',6'-bis(acetyloxy)-3-oxospiro[isobenzofuran-1(3H),9'-[9H]xanthene]-4',5'-diyl]bis(methylene)]bis[N-[2-

[(acetyloxy)methyoxy]-2-oxoethyl]-, bis[(acetyloxy)methyl] ester), a non-toxic, membrane-permeable, non-fluorescent dye that, upon uptake of living cells, is hydrolyzed by endogenous esterase into negatively charged calcein with green fluorescence at an excitation wavelength of 485 nm and emission wavelength of 530 nm.

3.2.9. DCFH-DA Assay. As discussed in Section 3.1.2, 2′, 7′-Dichlorofluorescin diacetate (DCFH-DA, CAS name: Benzoic acid, 2-[3, 6-bis(acetyloxy)-2,7-dichloro-9H-xanthen-9-yl]) was used in the intracellular ROS study. DCFH-DA is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species (ROS) in cells. Upon cleavage of the two acetate groups by intracellular esterases and oxidation, the non-fluorescent H_2DCFDA was converted to the highly fluorescent 2', 7'-dichlorofluorescein (DCF).

3.2.10. Determination of Reduced and Oxidized Glutathione. Reduced glutathione (GSH) is the principal endogenous thiol for scavenging free radicals and detoxification of xenobiotics. Upon oxidation, GSH is dehydrogenated and forms GSSG, a homologous dimer, as its oxidized form. The ratio of GSH/GSSG, therefore, is an important marker that indicates the extent of intracellular oxidative damage. Areversed-phase HPLC system was used for measuring intracellular GSH and GSSG levels.

3.2.10.1. GSH determination. The cell pellets collected were homogenized in SBB buffer (pH = 7.5). Approximately 1 mL of SBB was added to each 10^7 cell. The homogenate was centrifuged, clear supernatant was collected, and 50 µL of the supernatant were added to 200 µL of HPLC water and 750 µL of NPM (1mM in acetonitrile). The resulting solution was incubated at room temperature in darkness for 5 min, and the reaction was stopped by adding 10 µL of 2N HCl. The samples were

thereafter filtered through a 0.45 Om filter (Advantec MFS, Inc. Dulin, CA, USA) and injected onto HPLC system introduced in Section 2.2.4. The injection volume was 5 μL, and the mobile phase was 70%-30% (V/V) acetonitrile-water (HPLC grade) and was adjusted to pH 2.5 via adding 1 ml/L of both glacial acetic acid and o-phosphoric acid. The flow rate was 1 ml/minute.

3.2.10.2. GSSG determination. The levels of oxidized glutathione (GSSG) were determined via enzymatic reduction of GSSG via glutathione reductase (GR) using NADPH as substrate. In brief, the cell pellets were homogenized and centrifuged, as discussed in a previous section, and 95 μL of 2mg/mL NADPH and 5 μL of 5U/mL GR were added to 100 μL of the supernatant. After 5 min of incubation, 100 μL of the mixture were transferred to another test tube, to which 50 μL of HPLC water and 750 μL of NPM were added. The following procedure was the same as that in 3.2.10.1.

3.2.11. Determination of Malonaldehyde (MDA). The assay to determine the malondialdehyde (MDA) level relied on the nucleophilic attack of MDA by 2-thiobarbituric acid (TBA) and the formation of a 1:2 (MDA: TBA) adduct with intense red fluorescence at an excitation wavelength of 510 nm and an emission wavelength of 590 nm. In brief, the cell pellets were homogenized in SBB (pH = 7.5) and centrifuged. To 350 μL of supernatant, 550 μL of 5% trichloroacetic acid (TCA) and 100 μL of 500 ppm butylated hydroxytoluene (BHT) in methanol were added. The resulting solutions were then heated in a boiling water bath for 30 min, cooled on ice, and centrifuged. The supernatant fractions were mixed 1:1 with saturated TBA. The mixture was again heated in a boiling water bath for 30 min and cooled on ice. Afterwards, the adduct in 500 μL of each sample was extracted with 1 ml of n-butanol and centrifuged. The upper layers were

filtered through a 0.45 μm filter, and 200 μL of the filtrate were transferred to a 96-well plate for measurement of fluorescence.

3.2.12. Catalase (CAT) Activity Assay. The assay to determine CAT activity was based on the decreased absorbance of H_2O_2 at a wavelength of 240 nm due to its enzymatic decomposition. In brief, a 30% H_2O_2 stock solution was diluted in a potassium phosphate buffer (50 mM, pH =7.0), at a ratio of 3:1000 (v/v), to yield a working solution. The cell pellets were homogenized in the same buffer and centrifuged. A total 1.2 mL of supernatant was equally divided into two quartz cuvettes, with 400 μl of a working solution going to one and 400 μl of the buffer being added to the other. Using the latter one as a reference, the absorbance of the former one was immediately kinetically read at 240nm for 5 min using the spectrophotometer described in Section 2.2.7. One unit of CAT decomposed 1.0 μmole of H_2O_2 per minute with pH 7.0 at 25 °C. CAT activity was expressed in U/mg protein.

3.2.13. Glutathione Reductase (GR) Activity Assay. The assay of GR activity determination relied on the decrease in absorbance of NADPH, the substrate consumed during enzymatic reduction of GSSG by GR, at 340 nm. Briefly, the cell pellets were homogenized in a sodium phosphate buffer (50 mM with 1mM EDTA, pH = 7.8) and centrifuged. To 40 μL of a 25 mM GSSG solution in two cuvettes, 800 μL of the supernatant or buffer were added. To initiate reaction, 160 μL of 1.25 mM NADPH solution were quickly added and mixed. Using the latter one as a reference, the absorbance of the former one was immediately kinetically read at 340 nm for 5 min. One unit of GR oxidized 1.0 mmole of NADPH at pH 7.5at 25 °C. GR activity was expressed in U/mg protein.

3.2.14. Superoxide Dismutase (SOD) Activity Assay. The assay to determine total superoxide dismutase (SOD) activity relied on its inhibition of the reduction of oxidized cytochrome c by the $O_2^{\cdot -}$ (produced enzymatically by xanthine oxidase, XOD). This resulted in a slower decrease in the absorbance of cyt c at 550nm. In brief, a reaction cocktail (potassium phosphate buffer, 50mM, pH=7.8; 0.17mM EDTA; 0.017mM oxidized cyt c; 0.084mM xanthine) was freshly prepared. The cell pellets were homogenized in a potassium phosphate buffer (50mM, pH=7.8) and centrifuged, and 600 μL of the supernatant or buffer were mixed with 1.8 mL of cocktail and 600 μL of 0.01 U/mL XOD. Using the latter as reference, the absorbance of the former was immediately read kinetically for 5 min. One unit of SOD inhibited the rate of reduction of oxidized cyt c by 50% in a coupled system, using xanthine and XOD with pH 7.8 at 25°C. SOD activity was expressed in U/mg protein.

3.2.15. Studies of Mitochondrial Function. Mitochondrion is the major intracellular organelle for generation of ATP. Disruption of mitochondrial inner membrane potential due to oxidative stress might result in mitochondrial dysfunction, depletion of ATP, leakage of pro-apoptotic compounds (such as cyt c) and apoptosis.

3.2.15.1. Measurement of mitochondrial membrane potential. A novel cationic fluorescent dye, JC-1(CAS name: 1H-Benzimidazolium, 5,6-dichloro-2-[3-(5,6-dichloro-1,3-diethyl-1,3-dihydro-2H-benzimidazol-2-ylidene)-1-propenyl]-1,3-diethyl-, iodide, (E)-) was used to study mitochondrial membrane potential. This dye, at a low concentration, exists in cytosol as a monomer of a green fluorescence. At higher concentrations, however, it accumulates in the mitochondria and forms aggregates with a red fluorescence, which is only retained in the intact mitochondria. Therefore, JC-1 is a

useful tool for studying mitochondrial membrane potential. In brief, the cells were cultured in glass-bottom petri dishes and treated with NAC/NACA and specific oxidative drugs, as discussed in 2.1.3. The cells were washed twice with PBS and then incubated with JC-1 dye (1 μg/mL) in a medium for 30 minutes. The cells were then washed and placed under a fluorescence microscope in phenol-red-free SFM. The stained cells were observed with an Olympus IX51 inverted microscope at 400× total magnification with the microscope.

3.2.15.2. Mitochondrial superoxide anion ($O_2\cdot^-$) measurement.

Generation of mitochondrial superoxide anion ($O_2\cdot^-$) by NFT and effect of prevention by NAC/NACA was evaluated by MitoSOX Red Assay. MitoSOX Red is a fluorogenic dye of high selectivity of mitochondrial ($O_2\cdot^-$) in living cells; it is oxidized specifically in mitochondria by ($O_2\cdot^-$) to a compound of red fluorescence. In brief, the cells were seeded at a density of 1×10^4 cells/well in 96-well plate. After 24hrs of growth in complete medium, the cells were pretreated with various concentrations of NAC/NACA and then incubated with 5μM of MitoSOX Red working solution for 1hr in darkness. After washed twice with serum-free medium and treatment of optimum concentration of NFT for 1 hour, fluorescence was measured with an excitation wavelenth of 510nm and emission wavelength of 580nm using a microplate reader. Serum-free medium was used as negative control.

3.2.15.3. Determination of mitochondrial GSH. Mitochondria of HepaRG cells were isolated via gradient centrifugation. In brief, harvested cells were homogenized in HEPES buffer in glass grinder and the crude homogenate was centrifuged at 700g for 10min; then the supernatant was centrifuged at 10000g for 10min, and the pellet

(mitochondria) was collected, suspended in SBB, pH 7.5. Mitochondria were homogenized by electric homogenizer to release matrix GSH, and GSH content was determined as described above.

3.2.15.4. Measurement of total cellular ATP level. Total cellular ATP level was measured by using a luminescent ATP detection assay kit. The assay was based production of light caused by reaction of ATP with added firefly (*Photinus pyralis*) luciferase and D-luciferin, and the emitted light is proportional to ATP concentration inside the cells. In brief, HepaRG cells were seeded on a 96-well plate in complete medium for 24 hours, followed by pretreatment of NAC/NACA or serum-free medium for 2 hours, and treatment of NFT or serum-free medium for another 24 hours. Afterwards, the treatment was replaced by 100μL of serum-free medium, and 50μL of detergent from the kit was added to each well and shaken at 700RPM for 5 minutes. After lysis of cells, 50 μL of reconstituted substrate/enzyme (D-luciferin/luciferase) was added, followed by another 5 minutes of shaking at 700RPM. The plate is then adapted in darkness for 10 minutes and the luminescence was measured.

3.2.16. Determination of Protein. Protein levels of the cell samples were measured by the Bradford method [87]. Bovine serum albumin was used as the protein standard.

3.2.17. Statistical Analysis. All reported values were represented as the mean ± S.D (n=4). Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA). Statistical significance was ascertained by one way analysis of variance, followed by Tukey's multiple comparison tests. Values of $p<0.05$ were considered significant.

3.3.RESULTS

3.3.1. Results of Toxicities of Medicinal Drugs and NAC/NACA on Cells. A dose-dependent decrease in cell viability was observed in A549 cells upon exposure to BLM for 24 hours (Figure 3.1), which was confirmed using a calcein AM assay. Based on the dose response relationship, a 200 μM concentration of BLM, which decreased cell viability by about 40%, was determined to be optimal in evaluating the protective effects of NACA, as well as for all of the other following experiments.

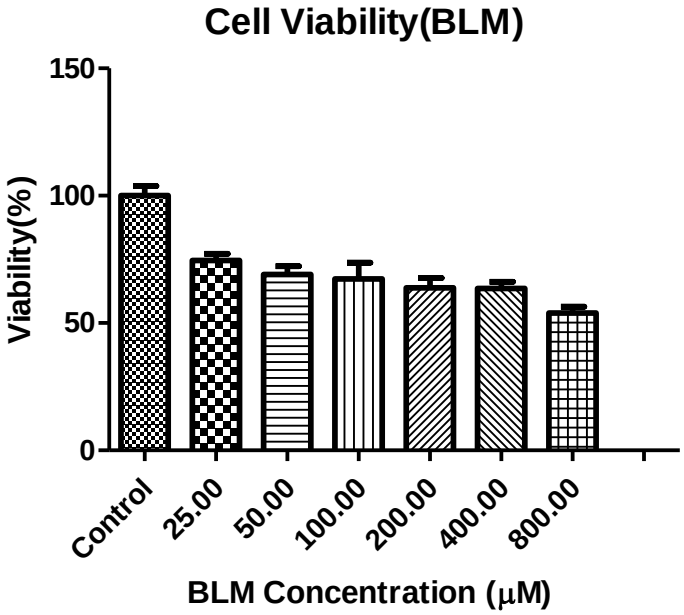

Figure 3.1. Dose-Dependent Toxicity of BLM on A549 Cells. Treatment with BLM decreases cell viability in a dose-dependent manner. A concentration of 200μM of BLM was found to cause ~40% of cell death, and was used as optimum dose for following experiments. The graph is representative of at least 3 independent experiments.

To assess the cytotoxicity of NACA, the A549 cells were incubated with different concentrations of NACA (0.10, 0.20, 0.50, 1.0, 2.0 and 5.0 mM) over a 24-hour period. NACA was nontoxic to A549 cells at concentrations lower than 2mM (Figure 3.2). Therefore, the highest non-toxic concentration of 2mM NACA was determined to be the optimal concentration for further experiments.

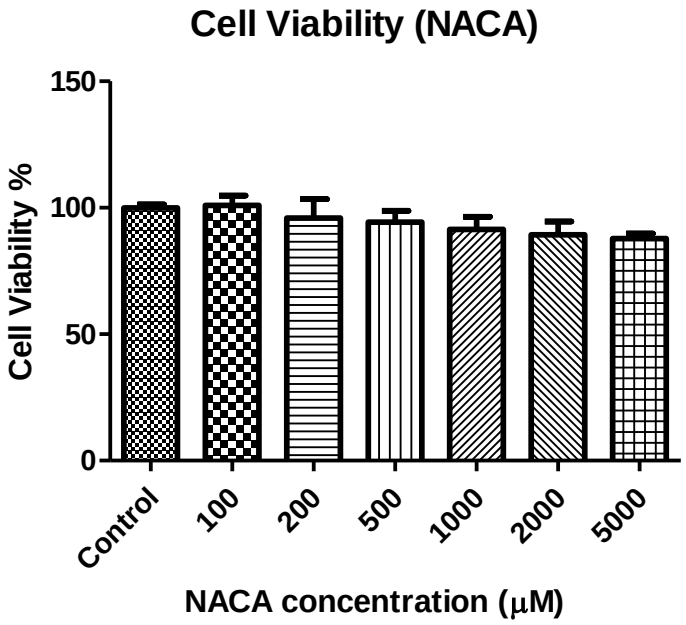

Figure 3.2. Dose-Dependent Toxicity of NACA on A549 Cells. A549 cells were treated with various concentrations of NACA (0.1 mM – 5.0 mM). Cell viability was quantified by Calcein AM after 2 hours of treatment. A concentration of 2mM NACA was found to be the highest non-toxic dose and was used for the rest of the experiments. The graph is representative of at least 3 independent experiments.

Similarly, a dose-dependent decrease in cell viability was observed in HepaRG cells exposed to NFT for 24 hours (Figure 3.3), which was confirmed using a calcein AM assay. Based on the dose response relationship, a NFT concentration of 500μM, which decreased cell viability by about 42%, was determined to be optimal for evaluating the protective effects of NAC/NACA, as well as for all of the other following experiments.

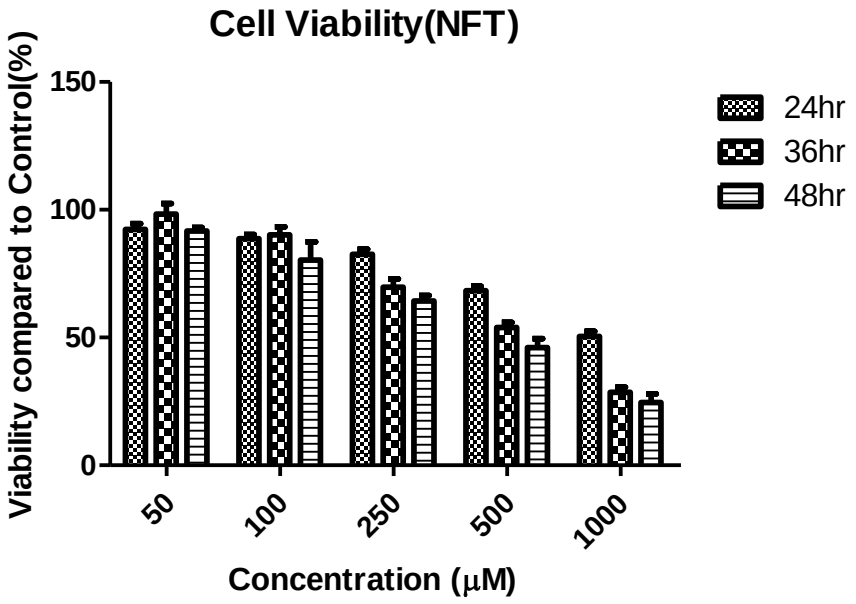

Figure 3.3. Time- and Dose-Dependent Toxicity of NFT on HepaRG Cells. NFT treatment decreases cell viability in a time- and dose- dependent manner. A concentration of 500μM was found to cause ~40% cell death in 24 hours, and was used as the optimum dose for all following experiments. The graph is representative of at least 3 independent experiments. To assess the cytotoxicity of NAC/NACA, the HepaRG cells were incubated with different concentrations of NAC or NACA (0.10, 0.20, 0.50, 1.0, 2.0 and 5.0 mM) over a 24 hour period. Both of them were shown non-toxic to HepaRG cells at all concentrations lower than 1mM, and NACA showed slightly lower toxicity than NAC at most concentrations. Therefore, the highest non-toxic concentration of 1mM NAC/NACA was determined to be the optimal concentration for further experiments.

To assess the cytotoxicity of NAC/NACA on HepaRG cells, they were incubated with different concentrations of NAC/NACA (0.10, 0.20, 0.50, 1.0, 2.0 and 5.0 mM) over a 24-hour period. As shown in Figure 3.4., the highest non-toxic concentrations of both NAC and NACA were found to be 1mM. Therefore, this was used as optimal concentration of NAC/NACA for the following experiments.

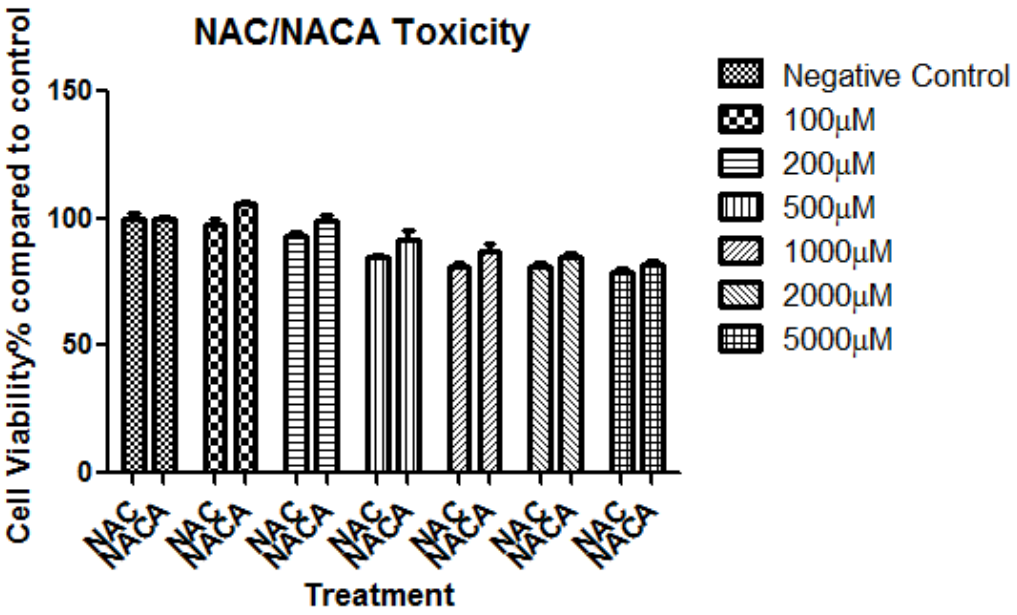

Figure 3.4. Dose-Dependent NAC/NACA Toxicity on HepaRG Cells. HepaRG cells were treated with various concentrations of NAC or NACA (0.1 mM – 5.0 mM). After 2 hours of treatment, cell viability was quantified by Calcein AM. A concentration of 1.0 mM of both NAC and NACA were found to be highest non-toxic dose, and were used for the rest of the experiments in the study. The graph is representative of at least 3 independent experiments.

3.3.2. Dose-Dependent Protection of NAC/NACA. To study the protective effects of NAC/NACA on BLM-induced cytotoxicity, A549 cells were pretreated for 2 hours with NAC or NACA of various concentrations (0.10, 0.20, 0.50, 1.0, 2.0, and 5.0 mM), followed by incubation with 200 μM of BLM for 24 hours (Figure 3.5). It was observed that both NAC- and NACA-pretreated A549 cells had significantly higher viabilities with BLM stress as compared to non-pretreated cells, and both pretreatments(up to 5mM) rendered dose-dependent increases in cell viability.

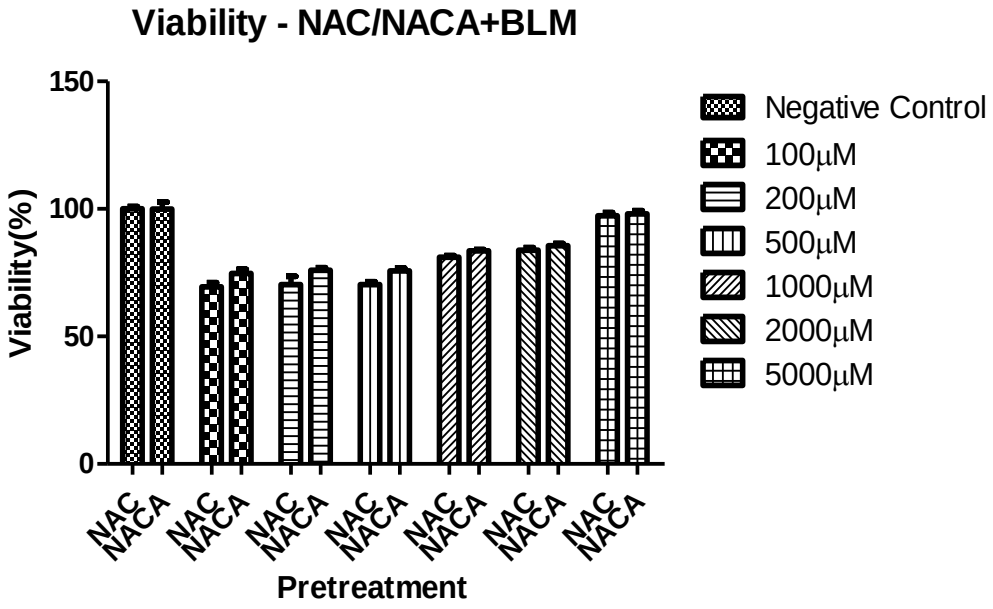

Figure 3.5. Dose-Dependent Protective Effects of NAC/NACA on BLM-Stressed A549 Cells. A549 cells were treated with various concentration of NAC or NACA, followed by treatment of 200 μM of BLM. Cell viability was quantified by Calcein AM assay. Both NAC and NACA pretreatments were found to protect against BLM toxicity in dose-dependent manners. NACA pretreatment resulted in higher cell viability below 1.0 mM. The results are representative of at least 3 independent experiments.

To compare the protective effects of NAC/NACA on NFT-induced cytotoxicity, HepaRG cells were pretreated for 2 hours with NAC/NACA of various concentrations, followed by incubation with 500 μM of NFT for 24 hours (Figure 3.6). The result showed a trend of a dose-dependent increase in viability of cells pretreated with NAC/NACA as compared to that without pretreatment. Also, cell viability values of the NACA-pretreatment groups were higher than those of the corresponding NAC-pretreated group.

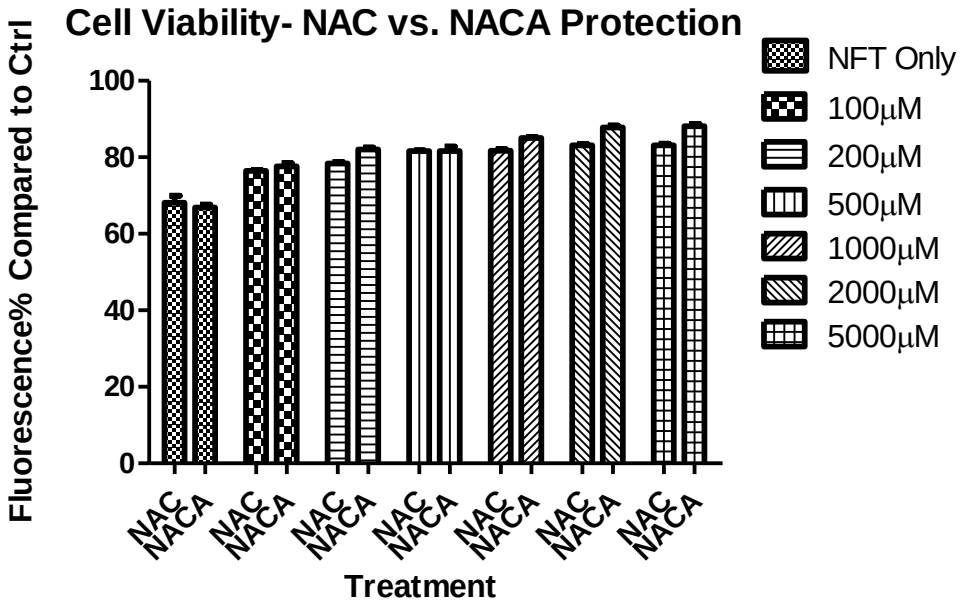

Figure 3.6. Dose-Dependent Protective Effects of NAC/NACA on NFT-Stressed HepaRG Cells. HepaRG cells were treated with various concentration of NAC or NACA, followed by treatment of 200 μM of NFT. Both NAC and NACA pretreatments were found to protect against NFT toxicity in dose-dependent manners. NACA pretreatment resulted in higher cell viability at all concentrations. The results are representative of at least 3 independent experiments.

3.3.3. Effect of NAC/NACA on Intracellular ROS. To substantiate the hypothesis that BLM cytotoxicity is related to oxidative stress, ROS levels were measured after the exposure of cells to BLM at various concentrations (25, 50, 100, 200, 400, and 800 μM) for 1 hour. As shown in Figure 3.7, a dose-dependent increase in the production of ROS in A549 cells was seen with exposure to BLM (at 200 μM, the optimal concentration, ROS was ~610% compared to control, but increased to 1096% at 800 μM).

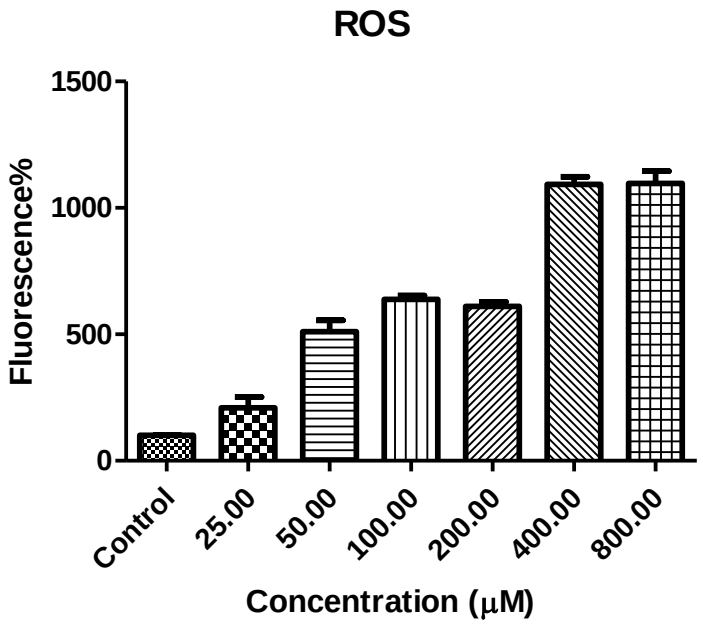

Figure 3.7. Dose-Dependent ROS Generation of BLM on A549 Cells. One-hour treatment of BLM generates intracellular ROS in a dose-dependent manner. The results are representative of at least 3 independent experiments.

To study the protective effects of NAC/NACA on BLM- induced increases in ROS levels, A549 cells were pretreated with NAC/NACA at various concentrations (0.10, 0.20, 0.50, 1.0, 2.0, and 5.0 mM) for 2 hours, followed by incubation with 200 μM of BLM for 1 hour. As shown in Figure 3.8, pretreatment with NAC/NACA decreased intracellular ROS, induced by BLM, in a dose-dependent manner up to 5 mM (54% for NAC-pretreatment and 48% for NACA-pretreatment, compared to non-pretreated cells).

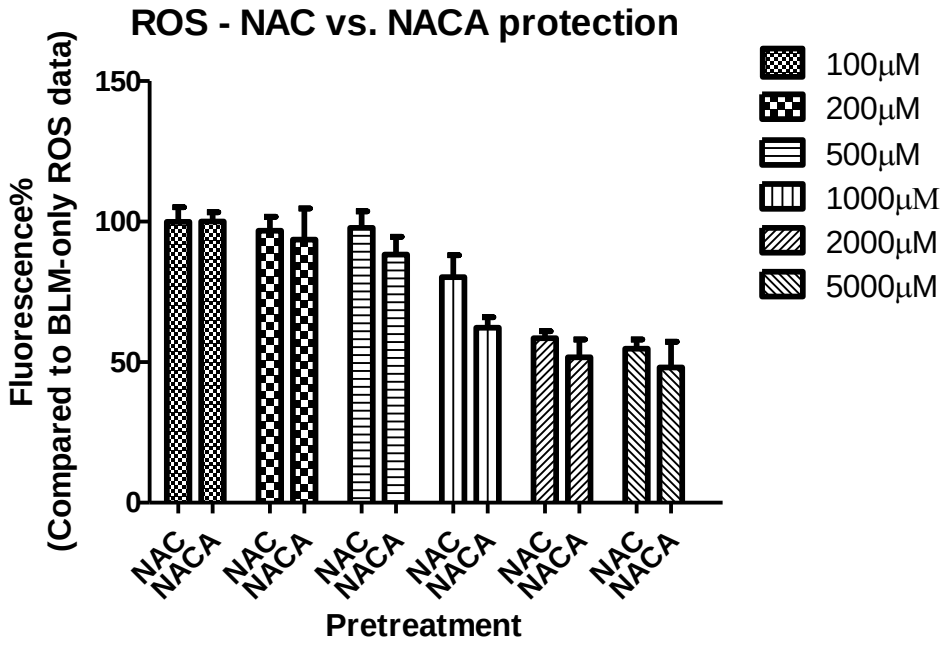

Figure 3.8. Dose-Dependent ROS-Scavenging Effects of NAC/NACA on BLM-Stressed A549 Cells. A549 cells were pretreated with NAC or NACA of various concentration (0.1 mM -5.0 mM) for 2 hours, followed by treatment of 200 μM of BLM for 1 hour. Both pretreatments decreased intracellular ROS level in dose-dependent manners. NACA pretreatments resulted in lower ROS level at all concentrations. The results are representative of at least 3 independent experiments.

To substantiate the hypothesis that NFT causes cell death through oxidative stress and to evaluate the protective effects of NAC/NACA, ROS levels were measured after cells were pretreated with NAC/NACA at various concentrations (0.10, 0.20, 0.50, 1.0, 2.0, and 5.0 mM) followed by treatment with 500 µM of NFT for 1 hour. As shown in Figure 3.9, incubation of 500 µM of NFT alone increased the intracellular ROS level to about 1150% of control; however, pretreatment with NAC/NACA decreased ROS in a dose-dependent manner, illustrating their antioxidant nature.

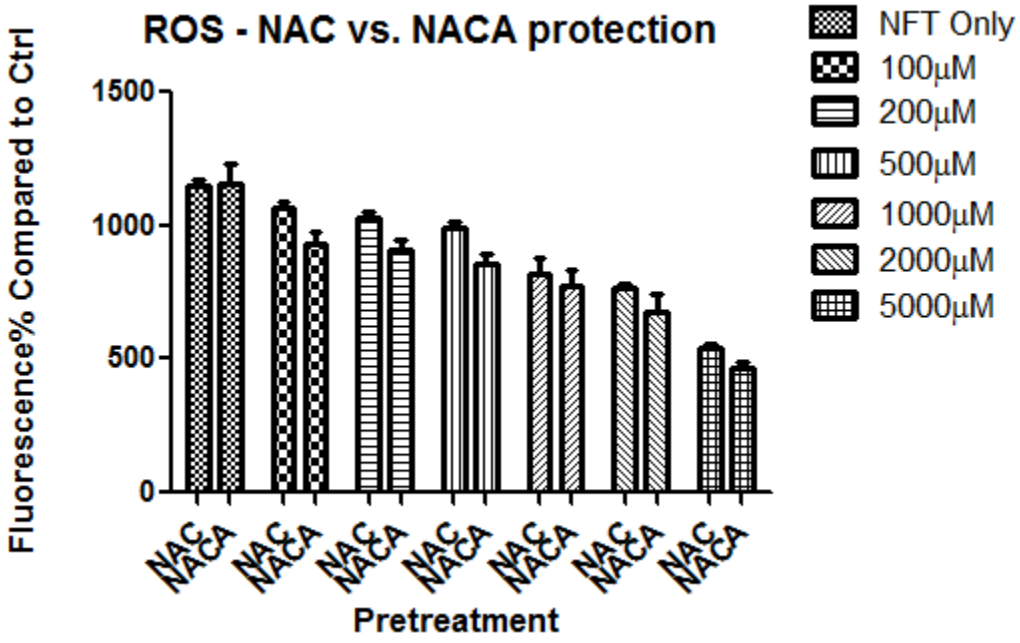

Figure 3.9. Dose-Dependent ROS-Scavenging Effects of NAC/NACA on NFT-Stressed HepaRG Cells. HepaRG cells were pretreated with NAC or NACA of various concentration (0.1 mM -5.0 mM) for 2 hours, followed by treatment of 500 µM of NFT for 1 hour. Treatment with NFT alone significantly increased intracellular ROS, whereas both NAC and NACA pretreatments decreased intracellular ROS level in dose-dependent manners. NACA pretreatments resulted in lower ROS level at all concentrations. The results are representative of at least 3 independent experiments.

3.3.4. Effects of NAC/NACA on Intracellular Glutathione Levels. To further elucidate the mechanism by which BLM induces cell death and damage, its effects on intracellular levels of reduced glutathione (GSH), the main endogenous antioxidant defending oxidative stress, were studied. This is one of the most important markers indicative of the extent of oxidative damage. Figure 3.10 shows the effect of BLM on cellular GSH levels in A549 cells in the presence and absence of NAC/NACA. A 24-hour exposure to 200 μM of BLM decreased the GSH level to 47% of that of the control, fortifying the hypothesis that oxidative stress was involved in BLM-induced cell damage. A treatment with 200 μM of BLM, with a 2 mM pretreatment of NACA, had results significantly different from those of the BLM only group, as well as the NAC+BLM group. GSH concentration in the NACA+BLM group was close to that of the control group. NAC significantly increased the GSH levels, when compared with the BLM-only group; however, NACA was more successful than NAC at increasing GSH levels to close to that of the control group (~87.0% of control for the NACA+BLM group, compared to ~76.6% of control for the NAC+BLM group). Besides, cells in both the NAC- and NACA-only groups had slightly higher GSH levels than those of the control group, and the NACA-only group had an even higher GSH level than the NAC-only group (~109.5% compared to ~102.7%), indicating that NAC/NACA scavenged the endogenous ROS and/or favored the intracellular *de novo* synthesis of GSH upon entering the cells, and that NACA had higher bioavailability than NAC.

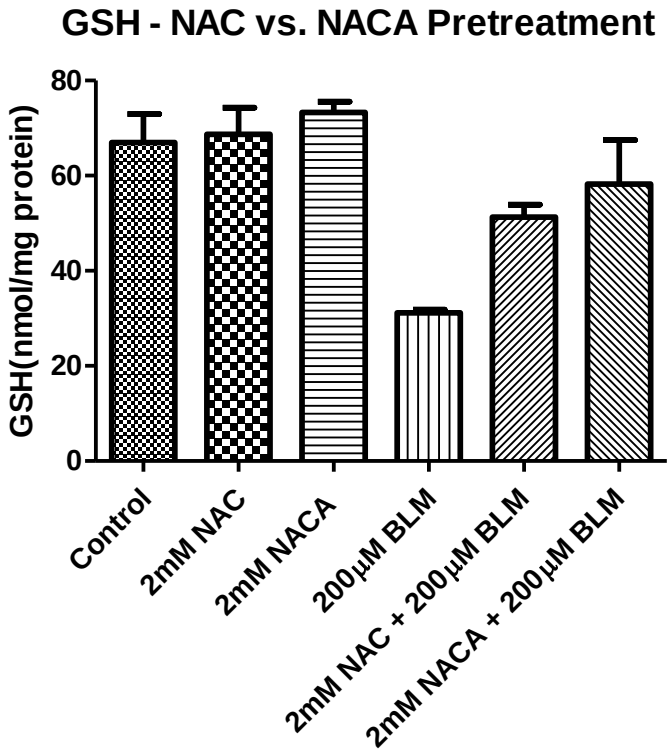

Figure 3.10. NAC/NACA Effects on Intracellular GSH Level on BLM-Stressed A549 Cells. A549 cells were pretreated for 2 hours with serum-free medium, 2mM of NAC or NACA, followed by treatment for 24 hours of 200 μM of BLM or serum-free medium. GSH levels were measured after all treatments. Exposure to BLM decreased intracellular GSH level to below 50% of control level, whereas both NAC and NACA pretreatment prevented such a dramatic decrease. NACA pretreatment resulted in ~10% higher GSH level than NAC. At least three independent experiments were performed.

In studies of NFT-induced oxidative stress in HepaRG cells, the effects of NFT on both reduced and oxidized glutathione (GSH/GSSG) were investigated. Figure 3.11 and 3.12, respectively, show the effect of NFT on cellular GSH and GSSG levels in HepaRG cells in the presence/absence of NAC/NACA. A 24-hour treatment of 500μM NFT alone decreased the GSH level to 45% of the control value and correspondingly increased the GSSG level to 170% of control, indicating the role of oxidative stress in NFT-induced cell damage. Pretreatment by NAC/NACA increased the GSH level back to 68% and 88%, as well as decreasing the GSSG level back to 128% and 103%, respectively. This shows the better protective effect of NACA, as compared to NAC, in maintaining an intracellular ratio of the reduced and oxidized glutathione level. This conclusion was further supported by results of the determination of both GSH and GSSG levels for the NAC-/NACA-only groups, since 2mM NAC or NACA pretreatments increased the intracellular GSH level to 103% or 107%, and decreased the intracellular GSSG level to 52% or 25% of control, respectively.

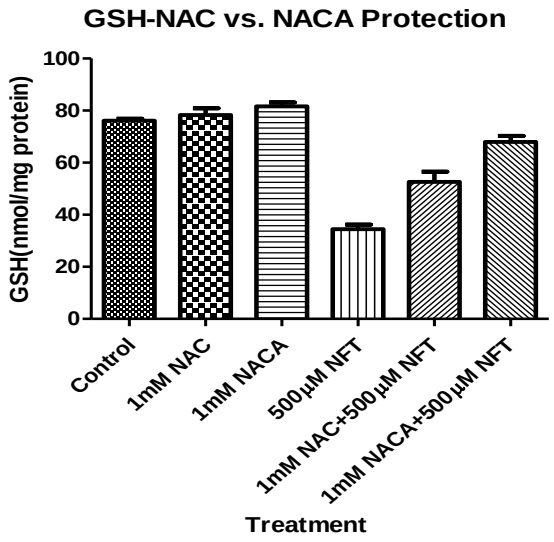

Figure 3.11. NAC/NACA Effects on Intracellular GSH Level on NFT-Stressed HepaRG Cells. Pretreatment of both NAC and NACA prevented depletion of GSH. NACA has significantly higher efficacy than NAC. At least three independent experiments were conducted.

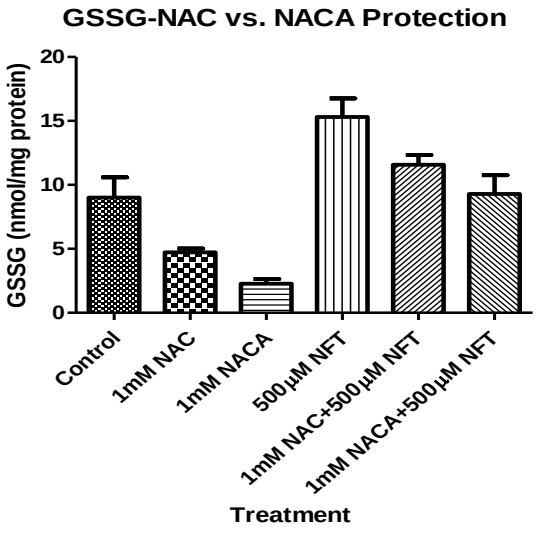

Figure 3.12. NAC/NACA Effects on Intracellular GSSG Level on NFT-Stressed HepaRG Cells. GSSG level significantly increased after treatment of NFT alone, whereas both NAC and NACA pretreatment prevented accumulation of GSSG. At least three independent experiments were performed.

3.3.5. Protective Roles of NAC/NACA against Lipid Peroxidation. The level of malondialdehyde (MDA), a main by-product during the process of lipid peroxidation, was also used as an index to oxidative damage. The effects of NAC and NACA on MDA levels in BLM-treated A549 cells were studied. Figure 3.13 shows that treatment with BLM alone resulted in a dramatic increase in the MDA level, which was 226% of control; whereas pretreatment with 2mM of NACA almost completely reversed the trend.

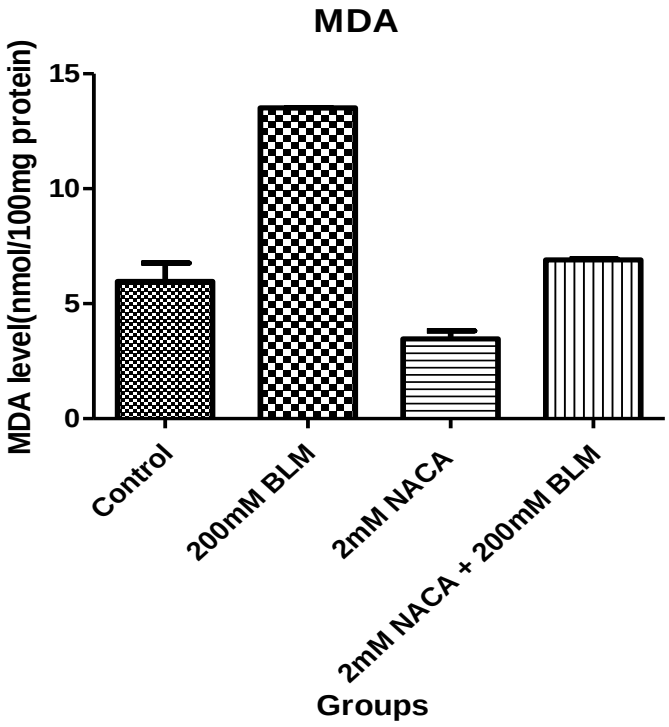

Figure 3.13. Effect of NACA on MDA Level on BLM-Stressed A549 Cells. It was found that MDA levels significantly increased after 24 hours of BLM treatment. MDA levels in NACA-only group were lower than those of control level. Pretreatment of NACA effectively prevented MDA generation upon BLM treatment. The graph is representative of at least 3 independent experiments.

In studies of NFT-induced oxidative stress in HepaRG cells, the effects of NAC/NACA on MDA levels were compared. As shown Figure 3.14, treatment with 500μM NFT for 24 hours increased the MDA level in HepaRG cells to 338% of the control level, whereas pretreatment with 2mM NAC or NACA reduced it to 242% or 187% of control, respectively. The NACA-pretreated group had significantly lower MDA levels as compared to the NAC-pretreated group, indicating the higher potency of NACA in preventing lipid peroxidation.

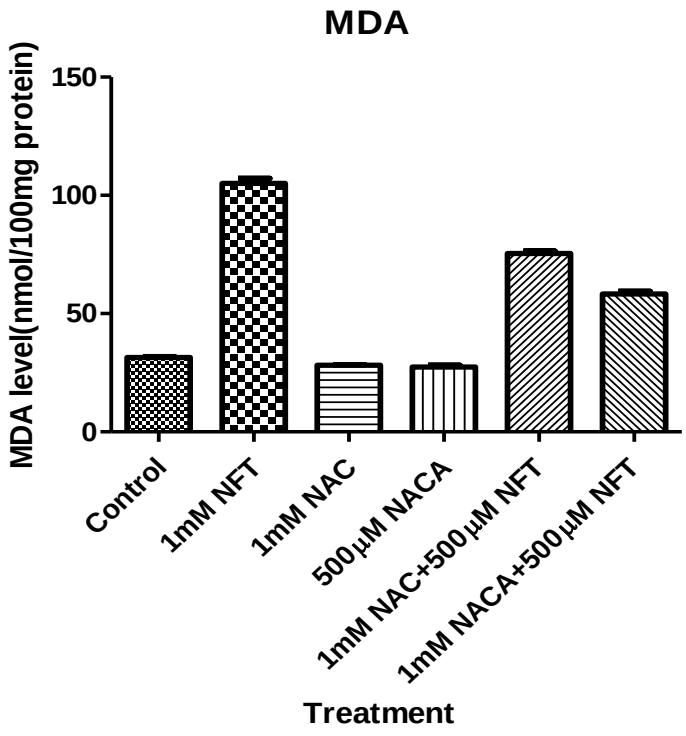

Figure 3.14. Effects of NAC/NACA on NFT-Stressed HepaRG Cells. Treatment of NFT dramatically increased intracellular MDA levels. The trend was prevented by both NAC and NACA pretreatment. NACA had more significant effect. The graph is representative of at least 3 independent experiments.

3.3.6. Effects of NAC/NACA on Activities of Antioxidant Enzymes. For evaluation of protective effect of NACA against BLM-induced oxidative stress, the activities of glutathione reductase (GR), catalase (CAT) and superoxide dismutase (SOD), which are the main endogenous antioxidant enzymes defending intracellular oxidative stress, were measured. The results are tabulated in Table 3.1. As shown, BLM treatment reduced activities of all three antioxidant enzymes but to a different extent, indicating that the oxidative stress induced by BLM might have overwhelmed the endogenous defensive system of these antioxidant enzymes. NACA treatment prior to BLM administration significantly reversed the trends for all enzymes, whereas incubation of NACA alone resulted in similar or slightly lower antioxidant enzyme activity as compared to the control. Underlying reasons are addressed in "Discussion" section.

Table 3.1. Effect of NACA on Antioxidant Enzyme Activity in BLM-Stressed A549 Cells. The values are representative of at least 3 independent experiments.

Groups	Glutathione Reductase	Catalase	Superoxide Dismutase
Control	100.00%	100.00%	100.00%
BLM-only	64.91%	23.71%	58.57%
NACA-only	100.53%	85.12%	101.81%
BLM + NACA	85.76%	80.01%	94.68%

In studies of the protective roles of NAC/NACA against NFT-induced oxidative stress in HepaRG cells, the activities of GR, CAT, and SOD after administration of NFT with or without pretreatment of NAC or NACA were measured. The results are tabulated in Table 3.2. While treatment of NFT lowered the activities of all antioxidant enzymes, the activity of CAT was lowered the most, indicating that CAT might be especially vulnerable to oxidative stress. Both NAC and NACA pretreatment, prior to NFT dosing, effectively prevented the loss of activities of these antioxidant enzymes, but NACA apparently had the higher efficacy. This was consistent with the results obtained from determination of the other oxidative stress markers listed in previous sections. It was interesting that, while treatment of an antioxidant alone seemed to lower CAT activity to some extent, activities of the other two antioxidant enzymes were hardly affected. This indicated that NAC/NACA might have effectively scavenged the endogenous H_2O_2 and, therefore, indirectly down-regulated the expression of CAT (this is covered in the "Discussion" section.)

Table 3.2. Effect of NAC/NACA on Antioxidant Enzyme Activities in NFT-Stressed HepaRG Cells. The values are representative of at least three independent experiments.

Groups	Glutathione Reductase	Catalase	Superoxide Dismutase
Control	100.00%	100.00%	100.00%
NFT-only	54.09%	37.41%	54.13%
NAC-only	100.70%	87.32%	101.14%
NACA-only	101.99%	83.88%	102.97%
NFT + NAC	75.48%	65.33%	82.58%
NFT + NACA	85.29%	82.23%	88.74%

3.3.7. Effects of NAC/NACA on the Mitochondrial Membrane Potential.

Mitochondria are an important intracellular target of oxidative drugs. A disrupted inner membrane of mitochondria could be either a source of intracellular ROS or, in turn, a result of oxidative damage (or both simultaneously), depending on the mechanism of the action of the xenobiotics.

To elucidate the effect of BLM on mitochondrial membrane potential, as well as the possible protective role of NACA, the membrane permeative potentiometric dye JC-1 was used. Cells with mitochondrial dysfunction showed primarily green fluorescence (which arose from the monomer of JC-1), whereas healthy cells with intact mitochondria (in which the dye molecules had accumulated and formed aggregates with a red shift in fluorescence) were differentiated by a green fluorescence as a background (cytosol) within which there are red fluorescent discrete spots (indicating mitochondria). As shown in Figure 3.15, control cells were stained both red and green, whereas a decrease in red fluorescence was seen in the BLM-treated group (as well as much more scarce cells, indicating the cytotoxic effect of BLM). Decrease in the red fluorescence in the BLM-treated group indicated that the BLM treatment had disrupted mitochondrial membrane potential in the A549 cells. However, NACA pretreatment restored the mitochondrial membrane potential as evidenced by the red fluorescence similar to that of the control. These results support the hypothesis that NACA pretreatment restores mitochondria membrane potential and therefore preserves mitochondrial function.

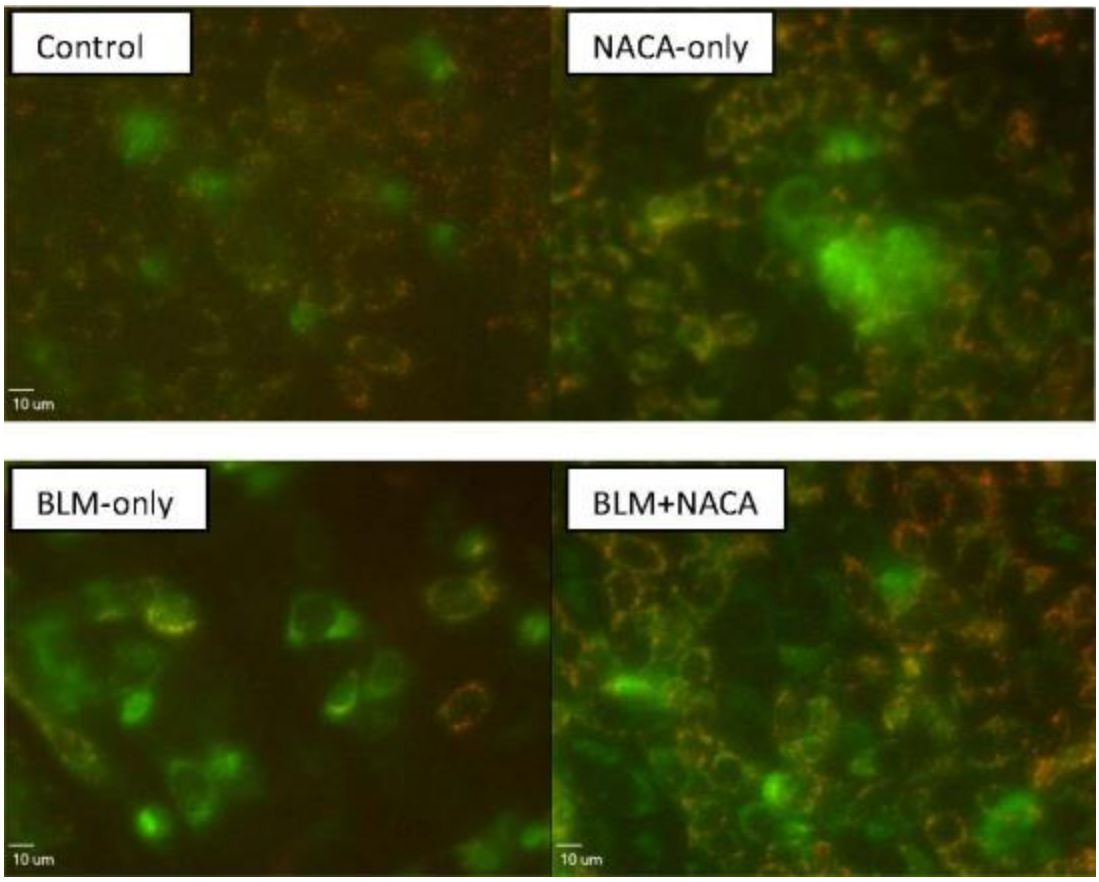

Figure 3.15. Effect of NACA on Mitochondrial Membrane Potential on BLM-Stressed A549 Cells. Green fluorescent patches indicated stained cells and red dots showed intact mitochondria. Treatment with BLM alone significantly decreased cell density and mitochondrial membrane potential. Pretreatment of NACA prevented the dysfunction.

NFT exerts its oxidative effect via redox cycling, which is catalyzed by various oxidoreductases, including mitochondrial ETC complex I and III. Therefore, it is reasonable to hypothesize that mitochondrial level of $O_2 \cdot^-$, the primary ROS produced in redox cycling, would significantly increase upon NFT treatment. MitoSOX Red, a mitochondrion-selective fluorescent dye to study the effect of NFT and NAC/NACA on mitochondrial $O_2 \cdot^-$ level, to which the intensity of red fluorescence from the $O_2 \cdot^-$ oxidative product of MitoSOX Red would be proportional, was used in the study. As shown in Figure 3.16, NFT treatment resulted in a dose-dependent increase of mitochondrial $O_2 \cdot^-$ level. NAC/NACA pretreatment, meanwhile, reversed the trend in a dose-dependent manner, as shown in Figure 3.17. The mitochondrial $O_2 \cdot^-$ levels in NACA-pretreated groups were lower than those in corresponding NAC-pretreated groups, implying the higher bioavailability of NACA.

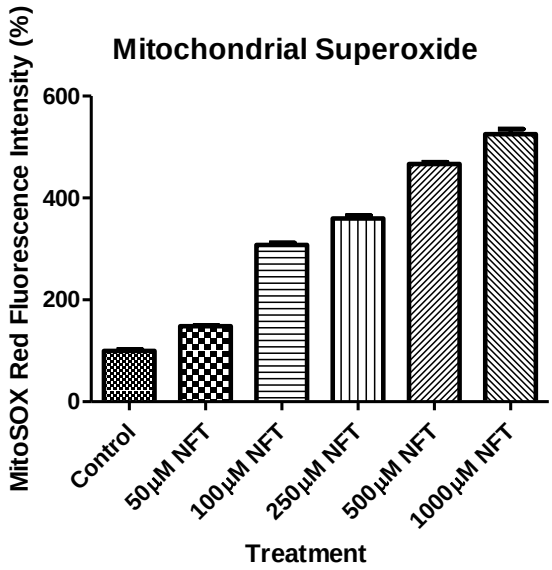

Figure 3.16. Dose-Dependent Effect of NFT on Mitochondrial $O_2\cdot^-$ Level on HepaRG Cells. HepaRG cells were treated with NFT of various concentration (50 μM – 1000 μM). NFT treatment increased mitochondrial $O_2\cdot^-$ level in a dose-dependent manner. At least three independent experiments were performed.

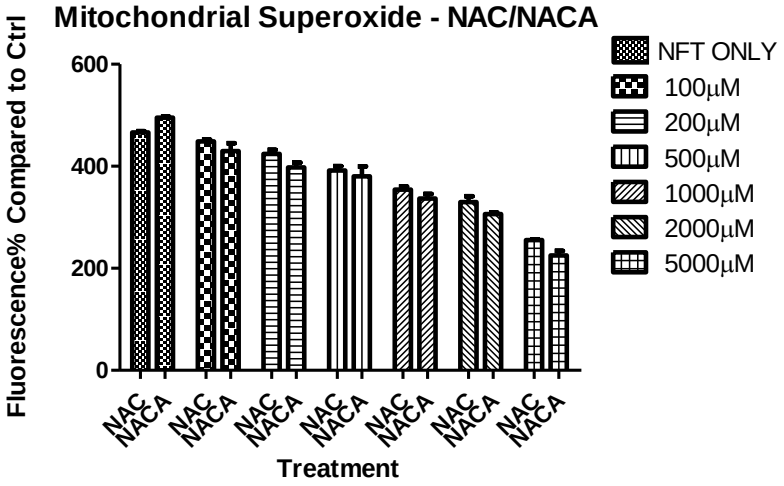

Figure 3.17. Dose-Dependent Effects of NAC/NACA on Mitochondrial $O_2\cdot^-$ Level on NFT-Stressed HepaRG Cells. HepaRG cells were pretreated with NAC or NACA of various concentration (0.1 mM – 5.0 mM), followed by treatment of 500 μM NFT. Treatment of NFT alone caused significant increase in mitochondrial $O_2\cdot^-$ levels, which was prevented by pretreatment of NAC or NACA in a dose-dependent manner. At least three independent experiments were performed.

Generation of significant amount of $O_2 \cdot^-$ in mitochondria would cause "local" oxidative stress and, expectably, decrease of mitochondrial GSH level. As shown in Figure 3.18, 24-hour treatment of NFT decreased mitochondrial GSH to 45% of control level, whereas pretreatment of both NAC and NACA significantly restored the depleted GSH (back to 69% and 86%, respectively). The fact that NACA pretreatment rendered higher mitochondrial GSH indicated its higher efficacy.

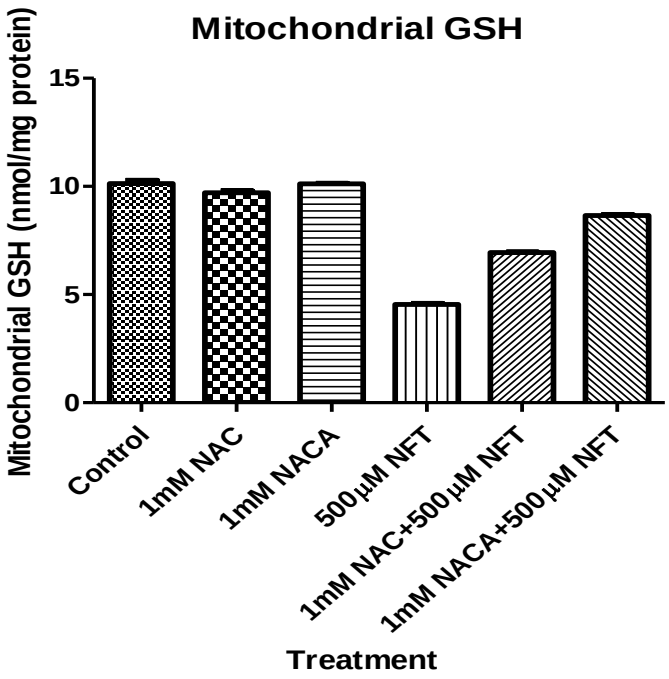

Figure 3.18. Effects of NAC/NACA on Mitochondrial GSH Level on NFT-Stressed HepaRG Cells. Treatment of GSH decreased mitochondrial GSH level to lower than 50% of control level. Both NAC and NACA pretreatment prevented depletion of mitochondrial GSH, and NACA was more effective. At least three independent experiments were performed.

Based on the knowledge that NFT disrupts function of mitochondrial ETC, it is reasonable to extrapolate that cellular level of ATP, the major intracellular energy "currency" that is primarily generated during oxidation-phosphrylation in ETC, would be negatively affected upon treatment of NFT. A luminescent assay was used to study effects of NFT and NAC/NACA on total cellular ATP. The intensity of luminescence would be proportional to ATP level. As shown in Figure 3.19, a 24-hour treatment of NFT almost completely depleted intracellular ATP, whereas pretreatment of NAC and NACA both prevented the trend of depletion, and NACA was of higher effect than NAC.

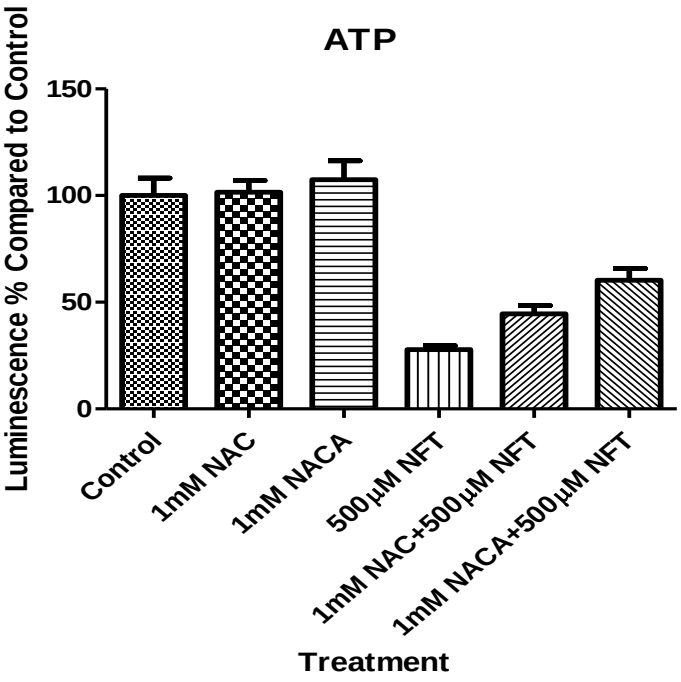

Figure 3.19. Effects of NAC/NACA on Cellular ATP Level on NFT-Stressed HepaRG Cells. ATP was depleted upon NFT treatment, but the trend was partially reversed by NAC or NACA pretreatment. At least three independent experiments were performed.

3.4. DISCUSSION

Oxidative stress, which is currently believed to be at least partially involved in a variety of pathogenesis and xenobiotic toxicity, occurs when reactive oxygen species (ROS) overpowers endogenous antioxidant defenses. Therefore, it is reasonable to assume that supplementation of a bio-compatible antioxidant would help alleviate oxidative damage. Reduced glutathione (GSH) is found to be the pivotal intracellular antioxidant compound; it was not feasible, however, to directly administer GSH to patients in cases of oxidative stress, because of the short half-life of GSH. Instead, N-acetylcysteine (NAC), a non-toxic synthetic thiol antioxidant, served as a precursor of the de-novo synthesis of GSH and also scavenged intracellular free radicals per se. Therefore, NAC, a mucolytic agent as well as an antioxidant diet supplement, has been used clinically as an antidote for acetaminophen overdose. The negative charge on the carboxylic group of NAC at physiological pH, however, makes it difficult for NAC to freely penetrate cell membrane. N-acetylcysteineamide (NACA), a novel thiol drug and an amide derivative of NAC, is therefore believed to be of higher bioavailability than NAC due to its neutrality. In the current study, the protective role of NACA against oxidative stress induced by two medicinal drugs in the cell was investigated.

Bleomycin (BLM), an antineoplastic drug, has not been widely applied clinically due to its pneumotoxicity. It induces pulmonary fibrosis (PF), an accumulative, irreversible and fatal pathologic process, in a dose- and time-dependent manner, and results in 3-5% mortality in patients. Toxicity of BLM is believed to be at least partially due to generation of ROS from redox cycling of the Fe(II)-BLM complex. This ROS, in

turn, would damage DNA, lipids and proteins and contribute to the loss of enzymatic activity and the structural integrity of epithelial membranes.

Nitrofurantoin (NFT), a member of nitrofuran antibiotics, is clinically applied for treatment of recalcitrant urinary tract infection and other types of infections caused by Gram-negative pathogenic bacteria, which are resistant to traditional β-lactam antibiotics. However, its application is limited due to its pulmonary and hepatic toxicity. It is believed that NFT exerts its toxicity by undergoing single-electron reduction on its nitro group, via catalysis of several intracellular oxidoreductases. The highly reactive intermediate derived from NFT could cause damage to biomolecules, either by direct attack or via superoxide anions generated in the oxidative half of a redox cycle.

Although there are concerns and debates regarding the "adverse effects" of antioxidant supplementation for the purpose of alleviating oxidative stress induced by "primary" medicinal drugs (i.e. toxicities of the antioxidants, attenuation of the therapeutic effects of primary drugs, as well as other possible drug interactions), supplements have always interested pharmacist and clinical doctors, and are reported to be beneficial to some extent. *In-vitro* studies were conducted to evaluate the protection efficacy of NACA against oxidative stress induced by the three medicinal drugs in their "target" cell lines, by measuring values of "oxidative stress markers". Results of this "preliminary" study will help support further *in-vivo* studies, and even provide valuable reference for future clinical application.

Clarification of the temporal sequence of the studies includes:

1. Protective role of NACA on BLM-stressed A549 cells

2. Protective roles of NAC/NACA on NFT-stressed HepaRG cells

Some modifications of methods used in some experiments were made during the studies. However, the principles for determining the main oxidative markers remained consistent, so it was still feasible to apply and interpret the results for the same markers in different studies.

3.4.1. Cytotoxicity of Oxidative Medicinal Drugs and NAC/NACA. First, the dose-dependent toxicities the two medicinal drugs were assessed in order to determine the appropriate doses with optimal concentrations of these drugs, at which 60%-70% viability was retained by the dosed cells after treatment. This was essential for an accurate evaluation of the protective effects of NAC and NACA. As expected, all three drugs studied showed significant cytotoxicity in a dose-dependent manner. It was noticed that the dose of BLM and NFT that caused 40% of cell death in A549 cells and HepaRG cells were 200μM and 500μM, respectively.

It is difficult to transversely compare the toxicity of different drugs in distinct cell lines or accurately convert the concentrations of these drugs in *in-vitro* studies to clinical doses for patients. The finding that BLM and NFT are toxic at considerably lower concentrations, compared to other “oxidative medicinal drugs” of different mechanisms of reaction, e.g. acetaminophen (the data of acetaminophen toxicity study would not be shown, since it belongs to another project). The fact was in accordance with trends reported in clinical findings. Both BLM and NFT were found to have significant toxicities for patients, even at therapeutic dose, whereas acetaminophen did not cause severe adverse effect (hepatotoxicity) until the patient was overdosed. This discrepancy

might be due to the differences in their mechanisms for inducing oxidative stress. BLM and NFT, as “redox-cycling” drugs, are considered to be “direct generators of ROS”. Theoretically, they act as “catalysts” for ROS (primarily $O_2 \cdot^-$) generation, and are capable of continuously generating ROS much more than one equivalent, as long as proper intracellular reducing agent (e.g. NADPH) is available. Acetaminophen and other CYP450-mediated “pro-oxidative” drugs, nevertheless, exert their oxidative effect mainly via excessive generation of reactive metabolite, which conjugates with intracellular GSH and depletes it, at most “stiochiometrically”. Therefore, “redox-cycling” drugs are usually of much higher oxidative toxicity than “CYP450-mediated” ones.

Thereafter, the toxicities of both NAC and NACA were assessed. NACA was not toxic for A549 cells, at a concentration of 2mM (NAC toxicities for A549 cells were not studied), whereas neither NAC nor NACA were significantly toxic for HepaRG cells with concentrations of 1mM. Therefore, 2mM of NACA as well as 1mM NAC/NACA were chosen as optimal pretreatment concentration for further studies to maximize the potential antioxidant effect of the compounds.

The efficacies of *in-vitro* protection of NAC/NACA against cell death were also studied. In general, both NAC and NACA pretreatment rendered significantly higher cell viabilities in all three studies, regardless of the mechanisms of action of these oxidative drugs. The results indicated that NAC/NACA exerted their “general” antioxidant effects, rather than inhibiting the oxidative pathway of specific pro-oxidative drugs. The protective roles would be further discussed in following. NACA-pretreated groups were also observed to have higher cell viability than the NAC-pretreated group following treatment with oxidative drugs, indicating that NACA has higher bioavailability.

3.4.2. Effects of NAC/NACA on Intracellular ROS. In this study, ROS-generating effects of the interested medicinal drugs and ROS-scavenging powers of NAC/NACA were both studied. Treatment of both BLM and NFT dramatically increased intracellular ROS instantly (1 hour after incubation) in corresponding cell line (610% and 1150% compared to control level, respectively). The results showed the high potential of "redox-cycling" drugs of generating ROS.

As expected, the pretreatment of cells with either NAC or NACA resulted in significantly lower quantities of ROS as compared to non-pretreated groups, verifying the antioxidant potential of NAC/NACA. Considering the mechanisms of action of NAC and NACA, it is reasonable to assume that, upon pretreatment, NAC and NACA accumulated in the cells and served as a "precursor pool" for de-novo synthesis of GSH, upon its depletion due to oxidative stress, as well as radical scavengers per se. It was found that NACA pretreatment had a greater effect in preventing ROS generation than NAC pretreatment did, which was in accordance with results of cell viability studies.

3.4.3. Effects of NAC/NACA on Intracellular GSH/GSSG. Reduced glutathione (GSH) is the major endogenous antioxidant thiol that participates in a variety of important intracellular reactions. This includes protective reactions such as direct scavenging of free radicals, enzymatic detoxification of xenobiotics via reduction by glutathione peroxidase or conjugation by glutathione S-transferase, and the maintenance of levels of other antioxidants (ascorbic acid, tocopherol, etc). In addition, protection is provided by chelation of free heavy metal ions and other metabolic and biochemical reactions such as DNA/protein synthesis, amino acid transport and regulation of enzyme activity. Therefore, it is critical for cell survival that sufficient intracellular GSH be

maintained. GSH and GSSG levels are important markers of the extent of oxidative stress.

In this study, the intracellular levels of GSH and GSSG with treatment of BLM or NFT and with or without pretreatment of NAC/NACA were studied. Intracellular GSH levels decreased dramatically (over 50%) after 24 hours of incubation for all three drugs, and the NAC/NACA-pretreatment-only groups showed even slightly higher GSH levels than those of the control groups. This indicated that both compounds served as "precursor pool" for de novo synthesis of GSH. As for the NAC/NACA + drug groups, the GSH levels were significantly higher than those of the drug-only groups.

It is interesting that the trends for these two indices may or may not be the same, but depend on the nature of the reactions of GSH participation in the stress of pro-oxidants. Upon administration of redox-cycling drugs, the ROS directly generated would be diminished via reduction (such as the reaction catalyzed by glutathione peroxidase), at the cost of oxidation of GSH and accordingly, generation of GSSG. Therefore, GSH is depleted and GSH/GSSG ratio would decrease dramatically. In cases of "CYP450 mediated" drugs, however, GSSG would not be the main fate of GSH, since most of the GSH would be consumed in reaction of conjugation (with the reactive CYP450 metabolite of the drugs) instead of oxidation. Therefore, the ratio of GSH/GSSG would not decrease as significantly as in treatment of redox-cycling drugs, since there is minimal increase in denominator.

3.4.4. Effects of NAC/NACA on Lipid Peroxidation. Generation of ROS and depletion of GSH set off a cascade of further oxidative damage, including oxidation of lipid, protein, and DNA. Lipid peroxidation is one of the key mechanisms of ROS that cause cell death, not only by disruption of cytoplasmic/organelle membranes, but the by-products of lipid peroxidation are deleterious also.

Malondialdehyde (MDA) is one of the stable products of degradation of polyunsaturated fatty acid and a main marker of oxidative damage. By nature, it is a highly reactive and electrophilic aldehyde, being capable of forming a covalent protein adduct and reacting with deoxyadenosine and deoxyguanosine of DNA, thereby exerting its mutagenic effect. Thus, MDA must be maintained at a low intracellular level.

In this study, the effects of NAC or NACA on MDA levels upon treatment with BLM and NFT were evaluated. Treatments with both oxidative drugs resulted in dramatic increases in intracellular MDA levels, which were effectively prevented by NAC/NACA pretreatment. Accumulation of MDA might be the result of depletion of GSH, the substrate for glutathione peroxidase (GPx), which is the main enzyme for peroxide detoxicification. NAC/NACA supplementation repleted the GSH pool for normal functioning of GPx and/or reacted with MDA, preventing its accumulation.

3.4.5. Effects of NAC and NACA on Activities of Antioxidant Enzymes. Endogenous antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), play pivotal roles in diminishing potentially deleterious reactive species, preventing damage of bio-molecules from attack by ROS, and regulating activities of redox-sensitive enzymes. In

contrast to the consistent trends of alterations in other oxidative markers (i.e. decrease in intracellular GSH as well as increase in MDA levels) upon exogenous oxidative stress, there are contradictory reports regarding the change of activities of antioxidant enzymes. In this study, activities of SOD, CAT, and GR in cells upon BLM and NFT treatment with/without NAC/NACA pretreatment were measured.

It was found that activities of all three enzymes decreased upon BLM and NFT incubation of corresponding cells, albeit to a different extent. Interestingly, in both studies, CAT activities decreased to the greatest extent (~2/3), whereas SOD and GR activities decreased moderately (~1/2). Decreases in antioxidant enzyme activities could be attributed to oxidation of their own redox-sensitive functional groups (e.g., the sulfhydryl group) at the reactive or allosteric site and/or loss of the central metal ion essential for reactivity. This was true for CAT upon oxidative stress, since it contains iron ion in its heme group attached to the peptide chain, as well as for sulfhydryl groups. On the other hand, activities of GR and SOD, decreased to a lesser extent since they were mainly affected by the former and latter factors, respectively. GR contains redox-sensitive $FADH_2$ and cysteinyl residues, whereas SOD contains transition metal ions in the central site. Both NAC and NACA pretreatment restored activities of all antioxidant enzymes, but NACA pretreatment was more effective.

3.4.6. Effects of NAC/NACA on Mitochondrial Function. As discussed in earlier sections, mitochondrion could either be a source or a target of ROS upon treatment with oxidative xenobiotics, depending on the mechanisms of action. In a physiological condition, mitochondria generate superoxide anions via electron leakage to molecular oxygen through the electron transport chain (ETC), located on the inner

membrane. Upon oxidative stress, the redox status of the complexes in ETC might be altered, resulting in more generation of superoxide anions. These excessive "endogenous" ROS, together with the exogenous ones, in turn attack lipids of mitochondrial membranes, as well as mitochondrial proteins and DNA. Since intactness of the mitochondrial inner membrane is essential for the normal function of ETC and generation of ATP, its disruption would result in the uncoupling of oxidative phosphorylation and an inadequate intracellular energy supply. Moreover, leakage of cytochrome c or other pro-apoptotic molecules from the mitochondria to the cytosol could also occur, switching on corresponding signaling pathways and eventually causing cell death. Therefore, it is critical for survival of cell survival that mitochondria remain intact and function properly.

In this study, JC-1 dye was used to measure the mitochondrial inner membrane potential ($\Delta\Psi m$) upon treatment of BLM with or without pretreatment of NAC/NACA. The results were observed via a fluorescent microscope. In spite of the distinct methods, loss of $\Delta\Psi m$ was observed upon BLM treatment, indicating that dysfunction of mitochondria is a general phenomenon upon oxidative stress. NAC and NACA both prevented the trend, showing their antioxidant potential.

It was found that NFT mainly and primarily boosts oxidative stress in mitochondrion and endoplasmic reticulum (ER) via single-electron reduction by cytochrome P450 reductase on membrane of ER and complex I/III in electron transport chain on inner membrane of mitochondria, respectively. The resulting active intermediate or $O_2\cdot^-$ would deplete GSH and other antioxidant in these organelles, which is extremely dangerous in case of mitochondrion, since it is the main source of intracellular ATP. Depletion of mitochondrial GSH not only renders inner membrane of mitochondria

vulnerable to further ROS attack, but also results in disruption of function of enzymes involved in mitochondrial signal transduction, due to oxidation of critical –SH groups of the enzymes. Both factors cause mitochondrial permeability transition (MPT) and in turn, decrease of mitochondrial membrane potential and ATP production. Therefore, the effect of NFT on mitochondrial function and corresponding protective roles of NAC/NACA were the focal point of this study. As expected, mitochondrial $O_2 \cdot^-$ increases as a concentration-dependent manner upon treatment of NFT and both mitochondrial GSH and cellular ATP was depleted, showing the effect of NFT in disruption of normal mitochondrial function. Pretreatment of both NAC and NACA effectively reversed these trends, displaying their protection on mitochondria from oxidative stress. NACA showed higher efficacy than NAC, indicating its higher bioavailability.

3.5. SUMMARY

In summary, the results showed that both medicinal drugs caused oxidative damage to cells in *in-vitro* studies, although the extent of alterations to some oxidative markers was different due to distinct mechanisms for induction of oxidative stress. Both NAC and NACA were proven to be effective protectors against oxidative damage, regardless of mechanisms. They both prevented excessive cell death, decreased intracellular ROS, replenished GSH, prevented MDA generation, and restored antioxidant enzyme activities, as well as mitochondrial inner membrane potential. NACA was found to demonstrate more antioxidant potency than NAC did, probably because of its higher bioavailability. Therefore, NACA showed its promising potential as a GSH

pro-drug and as a substitute for NAC. More experiments are needed, however, to determine the short-term and long-term toxicity of NACA, as well as its possible reactions with co-administrated drugs. These need to be conducted prudently before conclusions about its safety and efficacy can finally be ascertained. Future studies might focus on *in-vivo* assessment of NACA's antioxidant potential against oxidative stress induced by medicinal drugs. It will also be of interest to determine whether pre- or co-administration of NACA will attenuate the therapeutic effects of the main drugs. This must be determined case-by-case before NACA can be considered as a clinical drug or supplement that can be widely used.

4. PRELIMINARY STUDY: ANTIOXIDANT POTENTIAL OF *SUTHERLANDIA FRUTESCENS* IN CELL-FREE SYSTEM AND ITS *IN-VITRO* PROTECTION AGAINST OXIDATIVE STRESS

For past decades, there have been a number of "artificial" antioxidants synthesized and used in the food industry to prevent deterioration and rancidity of lipid-containing food due to the peroxidation of fatty acid. The most commonly used phenolic synthetic antioxidants include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroxyquinone (TBHQ), and propyl gallate (PG). Dietary or medical application of these artificial antioxidants, however, poses risks due to their toxicity, since they are believed to be carcinogenic in nature. Therefore, pharmacists are interested in seeking natural antioxidants among plants, especially those utilized historically as food, dietary supplements, or ethnomedicine. These natural antioxidants are believed to be of higher efficacy and lower toxicity than synthetic ones.

There are a great number of plants around the world that are used in folk medicine, including woody plants, herbs, algae, and mushrooms. Some of them have been found to have great versatility in medicinal effects, including antibacterial/antifungal/antiviral, anticoagulant, anti-inflammatory/antipyretic/analgesic, anticancer, astringent, and tonic effects. Many of these effects are found to be at least partially due to the antioxidant potential of the plants. There is considerable diversity in the metabolites of these plants that has proved to be responsible for their antioxidant effect, including phenolic compounds and their derivatives (such as tannins, flavonoids, phenolic acids/quinones, lignins, coumarins, betalains, etc.), nitrogen-containing compounds (alkaloids, chlorophyll, amines, amino acid and their derivatives, peptides, etc.), sulfur-containing compounds (mostly thiols), carotenoids, terpenoids, tocopherols,

ascorbic acid, etc. These metabolites are found to provide antioxidant activity due to their capability of reacting with free radicals, acting as $O_2^{\cdot-}$ scavengers, and chelating free catalytic metals.

Several types of medicinal drugs induce oxidative stress via various mechanisms and therefore, exert their adverse effects (collectively referred to as "oxidative drugs". These mainly include NSAIDs and acetaminophen, specific antimicrobial agents (sulfonamide, nitrofurans, and isoniazid), antimalarial drugs (quinones), and antineoplastic drugs (alkylating agents, anthracyclines, and bleomycin). The most commonly observed and potentially life-threatening adverse effect includes hemolytic anemia, especially in patients with certain types of inherited genetic disorders (such as G-6-PDD) that increase vulnerability to oxidative stress. Other effects are toxic epidermal necrolysis, neurotoxicity, and toxicity in other major organs (pulmonary fibrosis, cardiotoxicity, and hepatorenal toxicity). The oxidative-stress related adverse effects of these drugs not only render them contraindicated in patients with the genetic defects mentioned above, but also strike patients of normal genotypes and sometimes can even become dose-limiting factors upon administration, greatly restricting their clinical application. There have been comprehensive studies, therefore, in attempts to find possible solutions that can alleviate the oxidative damage induced with minimal attenuation by the main effects.

Although there have been concerns and conflicting reports of studies regarding decreases in the therapeutic efficacy of these oxidative drugs, co-administration of antioxidants has always been one of the most interesting strategies for pharmacists and clinical doctors. Specifically, the effects of "natural" antioxidants derived from plants

have been studied in the belief that their toxicity is lower and the therapeutic index is higher. In cases of anticancer therapies, for instance, it has been proved that supplementation of ascorbic acid, tocopherol, and carotenoids helps reduction of drug toxicity so that patients are more tolerant to treatment and are able to complete the desired regimen. There are also reports regarding the protective roles of these compounds against oxidative damage induced by other medicinal drugs. Straight extracts of some plants (most of which serve as ethnomedicines) that have also been assessed in a similar manner to determine their antioxidant potential have proved to be promising. However, more thorough *in-vivo* studies are needed regarding their toxicities (both short- and long-term), as well as possible cross-reactions with the main drugs, before they can be used clinically in the future.

Sutherlandia frutescens (SF) is a legume native to South Africa and has long been used as folk medicine. It has been found to be effective in prophylaxis and treatment for a variety of disorders, including bacterial/viral infection, Type II diabetes, cancer and inflammatory diseases. It was found that SF has antioxidant potential, which at least partially contributes to its therapeutic effects. Therefore, SF was interested due to its possible protective role against medicinal drug-induced oxidative stress.

The previous studies of components of SF plant material revealed that there were a great variety of its secondary metabolites that are potentially antioxidant, which makes it necessary to consider organic solvents as possible candidates for extraction of SF antioxidant components, for the sake of maximum efficiency. In the present study, six different ways of extraction (hot water/cold water/homogenization/methanol/ethanol/ acetone/acetonitrile) were performed, and the six resulting extracts were tested for their

antioxidant potencies. These included the content of total phenols and flavanoids (the two major antioxidant metabolites in plants), total reducing power, radical scavenging power (total, as well as several most commonly seen in living organisms), and iron chelating capability. Based on the results of antioxidant potencies identified in these extracts in a cell-free system, it was concluded that hot water extract was the most effective. For *in-vitro* studies, the extract was lyophilized and re-suspended in SFM, to test its protective role against t-butyl hydroperoxide (tBHP) in three cell lines (A549, CHO, and HepaRG). Although SF extract has been reported to have an antiproliferative effect against carcinoma cells, the present study found that, at a lower concentration (up to 1mg/mL), the extract does not present significant toxicity for the tested cell lines. On the other hand, pretreatment of these cells with the SF extract helped defend against tBHP-induced oxidative stress by repletion of intracellular GSH as well as decreasing intracellular ROS levels. The experimental results for both cell-free and cell-based studies led to the conclusion that SF is a good source as a potent natural antioxidant. The present study serves as a preliminary investigation prior to further assessment of the *in-vivo* antioxidant effectiveness of SF against medicinal drug-induced oxidative stress.

4.1. EXPERIMENTAL DESIGN

Experiment I – Assessment of the antioxidant potency of *Suthenlandia frutescens* extracts in various solvents

As introduced in the previous section, water as well as a variety of organic solvents were chosen as "candidates" for extracting the antioxidant components from SF.

In brief, leaf samples of SF were dried, minced, and sieved for further use. In the present study, extracts of the samples were prepared as follows:

(1) Extraction with boiling water;

(2) Addition of cold water (room temperature) followed by homogenization;

(3) Extraction with HPLC grade methanol (room temperature);

(4) Extraction with ethanol (room temperature);

(5) Extraction with acetone (room temperature);

(6) Extraction with acetonitrile (room temperature).

The extracts were prepared and diluted to the same extent, and the following determinations were made in a cell-free system to assess the antioxidant potency of each extract:

(1) Total phenolic content;

(2) Total flavonoid content;

(3) Total reducing power;

(4) Total free-radical scavenging power;

(5) Superoxide anion scavenging power;

(6) Hydroxyl radical scavenging power;

(7) Nitric oxide scavenging power;

(8) Hydrogen peroxide scavenging power;

(9) Free-iron chelating power.

The results were compared transversely, based on which "optimal" method of extraction would be chosen for further experiments in part II.

Experiment II: *In-vitro* antioxidant potential and toxicity assessment of SF extract in various cell lines

The purpose of this experiment was to assess the *in-vitro* effectiveness of the protection provided by the SF extract against exogenous oxidative stress. The "optimal" strategy of extraction that was determined based on results of experiment I were used. The solvent of the extract was removed and the dry residues were re-suspended in corresponding serum-free media, followed by sterilization for further use.

Three different cell lines were used in this experiment to test the extent of SF antioxidant protection:

(1) human pulmonary alveolar carcinoma (A549) cells;

(2) Chinese hamster ovary (CHO) cells;

(3) human hepatoma (HepaRG) cells.

Tert-butyl hydroperoxide (tBHP), an organic peroxide, was used to induce oxidative stress in the present study. The following experiments were conducted thereafter on all three cell lines:

(1) Toxicity of tBHP (for optimal concentration of tBHP in these cell lines, which corresponded to ~30-40% cell death)

(2) Toxicity of SF extract (for optimal concentration of SF in these cell lines. The highest non-toxic concentration was chosen for further experiments)

(3) SF extract dose-response studies of tBHP – stressed cells (pretreatment of cells with various concentrations of SF extract, followed by treatment of tBHP with optimal concentrations, and determination of cell viability)

(4) Study of dose-dependent intracellular ROS-scavenging potency of SF extract in different concentrations

(5) Determination of intracellular GSH/GSSG levels

4.2. MATERIALS AND METHODS

4.2.1. Chemicals. The human alveolar basal epithelial cells (A549) and human hepatoma cells (HepaRG) were obtained from Invitrogen. Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). All chemicals used were from Sigma (St. Louis, MO) and Fisher Scientific (Fair Lawn, NJ).

4.2.2. Preparation of Plant Extracts. Dried/minced leaves of *Sutherlandia frutescens* (family: Fabaceae/Leguminosa), obtained from Big Tree Nutraceutical, Fish Hoek, South Africa, were extracted by using six different solvents. These were boiling water, room temperature water (with homogenization), and HPLC grade methanol, ethanol, acetone, and acetonitrile. Briefly, 1g of the dried SF was extracted with 50 mL of solvent. Methanol, ethanol, acetone, and acetonitrile extracts were prepared by adding the respective solvent to a dry SF sample, followed by sonication for 20 min. The hot water

extract was prepared by boiling the SF sample for 20 min and then cooling to room temperature. The cold water extract was prepared by homogenizing the SF sample in water by a tissue tearor (Biospec Products) for 20 min. All extracts were vacuum filtered and stored thereafter at 4°C until use (20 mg/mL). For in vitro studies, the hot water filtrate was lyophilized for 72 hours in a Savant refrigerated vapor trap (RVT4104-180) and then dissolved in a serum-free medium to a final concentration of 1mg/mL stock solution, referred to as a SFE (a yield of 5%).

4.2.3. Determination of Total Polyphenolic Content. Total phenolic contents of the SF extracts were determined, as described by Konaté et al. with minor modifications [88], which relied on the formation of a bluish-grey complex between a Folin-Ciocalteu reagent (F-C reagent) with phenols. Briefly, 125 µL of the plant extract was mixed with 625 µL of a 10-fold diluted F-C reagent and incubated at room temperature for 5 min, followed by the addition of 500 µL of 75 mg/mL Na_2CO_3 solution. The mixture was vortexed and incubated at room temperature in darkness for 90 min. The absorbance at 760 nm was measured against a reagent blank. Gallic acid was used as the standard. The results were expressed as µg of gallic acid equivalents (GAE) / mg of dried plant leaves.

4.2.4. Determination of Total Flavonoid Content. Total flavonoid contents of the extracts were determined as described by Kalava et al. with minor modifications, which relied on the formation of a bright yellow complex between aluminum chloride and flavones/flavonoids [89]. Briefly, 200 µL of plant extract were mixed with a solution of 60 µL of 5% (w/V) $NaNO_2$ and 800 µL of HPLC water. The mixture was vortexed and allowed to stand at room temperature in the dark for 5 min. Thereafter, 60 µL of 10%

(w/V) $AlCl_3$ were added to the mixture, followed by 5 min of incubation at room temperature in the dark. The color was developed by adding 400 µL of 1 M NaOH. The absorbance at 415 nm was measured against a reagent blank. Quercetin was used as a standard. The results were expressed as µg of quercetin equivalents (QE) / mg of dried plant leaves.

4.2.5. Determination of Total Reducing Power. Total reducing powers of the SF extracts were determined as described by Jayanthi et al. with minor modifications, which relied on the formation of a dark blue complex [90]. Briefly, a solution comprising 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% (w/V) $K_3[Fe(CN)_6]$ were added to 1 mL of plant extract and vortexed. The mixtures were incubated at 50 ^{0}C in a water bath for 20 min and then mixed with 2.5 mL of 10% (w/V) trichloroacetic acid (TCA) and centrifuged at 6,000 RPM for 10 min. From the supernatant, 2.5 mL were transferred into tubes containing 2.5 mL HPLC water and 0.5 mL of 0.1% (w/V) ferric chloride ($FeCl_3.6H_2O$). The resulting solutions were well mixed and allowed to stand for 5 min in dark. The absorbance at 700 nm was measured against a reagent blank. Ascorbic acid was used as a standard. The results were expressed as µg of ascorbic acid equivalent (AAE)/ mg of dried plant leaves.

4.2.6. Determination of Total Radical Scavenging Power. The total radical scavenging powers of SF extracts were determined as described by Shyamala et al. [91] with minor modifications, which relied on disappearance of the deep purple color of 2,2-diphenyl-1-picrylhydrazyl (DPPH), a free-radical compound, upon its quenching by radical scavengers. In brief, 2.9 mL of 0.1mM DPPH were mixed with 0.1 mL of the SF extracts. The mixture was allowed to stand for 30 min in the dark. The absorbance at 520

nm was measured against a reagent blank. Decreases in intensity corresponded to a higher radical scavenging power, calculated as $[1-A_1/A_2]*100\%$, whereas A_1 and A_2 were the absorbance with and without plant extract, respectively. Butylated hydroxytoluene (BHT) was used as a standard.

4.2.7. Determination of H_2O_2 Scavenging Power. The H_2O_2 scavenging power of SF extracts was determined as described by Ozyurek et al. with minor modifications [92], which relied on formation of an orange-colored complex between neocuproine, a Cu(I)-specific chromogen, and Cu (I), the reduction product of Cu(II) by H_2O_2. In brief, 500 µL of phosphate buffer (200 mM; pH=7.4), 400 µL of 10 mM H_2O_2 or HPLC water, 200 µL of extract or solvent, and 400 µL of 0.1 mM $CuCl_2$ were mixed and incubated at 37 °C for 30 min. Then, 400 µL of HPLC water were added and 500 µL of this solution were added to a mixture of 1 mL of 10 mM $CuCl_2$, 1 mL of 7.5 mM neocupronine, and 2 mL of 1 M NH_4Ac. The absorbance at 450 nm was measured against a reagent blank. The increase in intensity of color corresponded to a higher H_2O_2 radical scavenging power, calculated as $[1-(A_1-A_2)/A_0]*100\%$, where A_0 was the absorbance of the mixture without the extract, but with H_2O_2; A_1 was that with both the extract and H_2O_2, and A_2 was that with the extract but without H_2O_2. Sodium pyruvate was used as a standard.

4.2.8. Determination of NO Scavenging Power. The NO scavenging powers of SF extracts were determined as described by Kumar et al. [93] with minor modifications, which relied on the formation of a rose-colored diazo compound between Griess Reagent and nitrate, the oxidation product of nitric oxide released by sodium nitroprusside. Briefly, 1 mL of the extract or solvent was mixed with 0.3 mL of 60 mM sodium nitroprusside, and illuminated under fluorescent light at room temperature for 2.5 hrs.

Thereafter, 0.5 mL of Griess Reagent [1% (w/V) sulfanilamide, 2% (w/V) phosphoric acid and 0.1% (w/V) N-(1-naphthyl)-ethylenediamine·2HCl] or HPLC water was added. The absorbance at 546 nm was measured against a reagent blank. The increase in intensity of the color corresponded to a higher NO scavenging power, calculated as [1-(A_1-A_2)/A_0]*100%, where A_0 was the absorbance of the mixture without extract, A_1 was the absorbance with both extract and Griess Reagent, and A_2 was the absorbance with extract and without Griess Reagent. Curcumin was used as a standard.

4.2.9. Determination of $O_2\cdot^-$ Scavenging Power. The $O_2^{\cdot-}$ scavenging power of the extract, determined as described by Bajpai et al. [94] with minor modifications, relied on reduction of nitroblue tetrazolium (NBT) to a purple product by $O_2^{\cdot-}$, that was generated via a redox cycle by 5-methyl-phenazinium methyl sulfate (PMS). In brief, 1 mL of 156 μM NBT (in 100 mM phosphate buffer, pH 7.4) or buffer alone, 1 mL of 486 μM NADH (in a buffer) or a buffer, and 100 μL of extract or solvent were mixed. Thereafter, 100 μL of 330 μM PMS (in a buffer) or a buffer alone were added and the mixture was incubated at room temperature for 5 min. The absorbance at 560 nm was measured against a reagent blank. Increased intensity in color corresponded to a higher $O_2^{\cdot-}$ scavenging power, which was calculated as [1-(A_1-A_2)/A_0]*100%, where A_0 was the absorbance of the mixture without the extract but with NBT/NADH, A_1 was the absorbance with the extract and NBT/NADH, and A_2 was the absorbance with the extract and without NBT/NADH. Quercetin was used as a standard.

4.2.10. Determination of ·OH Scavenging Power. The ˙OH scavenging powers of the extracts were determined as described by Kunchandy et al. [95] with minor modification, which relied on the formation of a red-colored complex between the

thiobarbituric acid (TBA) and the oxidation product of 2-deoxyribose by ˙OH. In brief, 100 µL of 28 mM 2-deoxyribose or HPLC water, as well as 500 µL of 20 mM phosphate buffer (pH 7.4), were added to 100 µL of the extract or solvent. Thereafter, 100 µL of 1 mM of $FeSO_4$, 100 µL of 1 mM EDTA tetrasodium salt, and 100 µL of 10 mM H_2O_2 were added to the mixture, which was incubated at 37°C for 1 hr. Then, 2 mL of 2.8% (w/V) TCA and 2 mL of 1% (w/V) TBA were added. This mixture was boiled for 15 min and allowed to cool to room temperature. The absorbance at 532 nm was measured against a reagent blank. Decreased intensity in color corresponded to a higher ˙OH scavenging power, which was calculated as $[1-(A_1-A_2)/A_0]*100\%$, where A_0 was the absorbance of the mixture without extract but with deoxyribose, A_1 was the absorbance with both extract and deoxyribose, and A_2 was the absorbance without deoxyribose but with the extract. Mannitol was used as a standard.

4.2.11. Determination of Iron (Fe^{2+}) - Chelating Power. The iron-chelating powers of SF extracts were determined as described by Ercal et al. with minor modifications, which relied on the formation of a magenta-colored complex of ferrous ion and ferrozine. [96] In brief, 100 µL of the extract (or solvent) were mixed with 100 µL of 0.6 mM $FeSO_4$ and 1.7 mL of HPLC water and incubated at room temperature for 5 min in the dark. Afterwards, 100 µL of a 5 mM ferrozine solution (in methanol) were added to the mixture and incubated for 5 min in the dark. The absorbance at 562 nm was measured against a reagent blank. Decreased intensity in color corresponded to a higher iron-chelating power, which was calculated as $[1-(A_1-A_2)/A_0]*100\%$, where A_0 is the absorbance of the control (without extract), A_1 is the absorbance in the presence of the

extract, and A_2 is the absorbance without ferrozine. EDTA tetrasodium salt was used as a standard.

4.2.12. Cell Culture. In brief, the human lung carcinoma pulmonary type II-like epithelium cells (A549) were seeded in 25 cm^2 flasks coated with type 1 rat tail collagen (Sigma-Aldrich, St. Louis, MO) and maintained in F-12 Ham's medium with 10% heat-inactivated fetal bovine serum in humidified 5% CO_2/95% air at 37 ^{0}C. The culture medium was changed every 3 days.

Human hepatoma cells (HepaRG) were seeded in 75 cm^2 flasks coated with type 1 rat tail collagen (Sigma-Aldrich, St. Louis, MO) and maintained in William's E medium supplemented with 10% FCS, 100 U of penicillin, 100 μg/mL of streptomycin, 5 ug/mL of insulin, and hydrocortisone in humidified 5% CO_2/95% air at 37 ^{0}C. The culture medium was renewed every 3 days. After about 2 weeks, when the cells were confluent, they were shifted to the same medium supplemented with 2% DMSO (differentiation medium). The medium was renewed every 2 to 3 days for 2 more weeks, and then switched to a DMSO-free medium for 1 day prior to the cells being used for assays.

Chinese hamster ovary (CHO) K1 cells were grown in Ham's F-12 culture medium, supplemented with 10% (v/v) fetal bovine serum (FBS), and 2 mM of L-glutamine, 100 U/mL of penicillin and 100 μg/mL of streptomycin. The cells were maintained in a humidified incubator at 37 ^{0}C and supplied with 95% O_2 and 5% CO_2.

4.2.13. Determination of Cell Viability. Cells were seeded in 96-well plates at a density of approximately 1.25×10^4 cells/well, for 24 hrs. To assess cytotoxicity of the

SF extract, the cells were incubated with various concentrations of SFE (10 µg/mL to 1 mg/mL) in a serum-free medium for 24 hrs.

To assess cytotoxicity of t-BHP, the media were replaced by various concentrations of t-BHP (10 µM to 500 µM) in a serum-free medium for 24 hrs. The concentration corresponded to approximately 40% cell death which will be used for further studies.

The protective effects of SFE were assessed by pretreating cells with various concentrations of SFE for 2 hours, followed by treatment with t-BHP for 24 hrs. Afterwards, the medium was discarded and viability was assessed with a Calcein AM assay kit (Biotium, Inc. CA). The cells were washed twice with PBS, and 100 µL of 2.0 µM Calcein AM in PBS were added to each well for 30 min at 37 °C. The fluorescence was measured with an excitation wavelength at 485 nm and an emission wavelength of 530 nm, using a microplate reader (FLUOstar, BMG Labtechnologies, Durham, NC, USA).

4.2.14. Measurement of Intracellular ROS Levels. Intracellular ROS generation was measured using a well-characterized probe, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). In brief, H_2DCF-DA was diffused into cells and deacetylated by cellular esterases to non-fluorescent 2', 7'-dichlorodihydrofluorescein, which was rapidly oxidized to highly fluorescent 2', 7'-dichlorofluorescein (DCF) by ROS. The fluorescence intensity was proportional to intracellular ROS levels.

In groups with SFE pretreatment, various concentrations of SFE were added to the cells seeded in a 96-well plate, followed by incubation for 2 hrs. Afterwards, the cells were washed twice with PBS and incubated with a working solution of 50 µM H_2DCF-

DA in a phenol-red-free medium for 30 min, followed by two PBS washings. The respective groups were then dosed either with t-BHP or serum-free medium for 24 hrs. Fluorescence was determined at 485 nm excitation and 520 nm emission, using a microplate reader (FLUOstar, BMG Labtechnologies, Durham, NC, USA).

4.2.15. Determination of Intracellular Glutathione (GSH) Levels. Intracellular GSH content was determined by reverse phase HPLC. The protective effects of SFE were studied by pretreating cells with an optimal concentration of SFE for 2 hrs, followed by treatment with t-BHP for 24 hrs. Cells were collected by trypsinization/centrifugation and the pellets were homogenized in pH 7.5 SBB. Thereafter, 20 µL of this homogenate were added to 230 µL of HPLC grade water and 750 µL of NPM (1 mM in acetonitrile). The resulting solutions were incubated at room temperature for 5 min. The reaction was stopped by adding 10 µL of 2 N HCl. The samples were filtered through a 0.45 µm filter (Advantec MFS, Inc. Dulin, CA, USA) and injected onto the HPLC system. For analysis, 5 µL of the sample were injected using a Thermo Finnigan TM Spectra SYSTEM SCM1000 Vacuum Membrane Degasser, Finnigan TM SpectraSYSTEM P2000 Gradient Pump, Finnigan TM SpectraSYSTEM AS3000 Autosampler, and FinniganTM SpectraSYSTEM FL3000 Fluorescence Detector (λ_{ex}=330 nm and λ_{em}=376 nm). The HPLC column was a Reliasil ODS-1 C_{18} column (Column Engineering, Ontario, CA, USA). The mobile phase was 70% acetonitrile and 30% water and was adjusted to a pH of 2.5 through the addition of 1 mL/L of both acetic and o-phosphoric acids. The NPM derivatives were eluted from the column isocratically at a flow rate of 1 mL/min.

4.2.16. Determination of Glutathione Disulfide (GSSG) Levels. Total glutathione content was determined by reverse phase HPLC. Cell samples were homogenized in SBB. Thereafter, 20 µL of this homogenate were added to 60 µL of NADPH (2 mg/mL) in nanopure water, and 20 µL of 1 unit/mL glutathione reductase were added to reduce GSSG. After 10 min of incubation at room temperature, 150 µL of H_2O were added, and the diluted samples were immediately derivatized with 750 µL of 1.0 mM NPM. The samples were analyzed by reverse phase HPLC, as detailed for the determination of GSH. Data from the original and total current GSH levels in each sample were subsequently used to calculate the levels of GSSG present in each sample.

4.2.17. Determination of Protein. Protein levels in the cell samples were determined using the Bradford method. Concentrated Coomassie blue (Bio-Rad, Hercules, CA, USA) was diluted 1:5 (v/v) with distilled water and 1.5 mL were added to 50 µL of diluted cell homogenate; the mixture was vortexed and allowed to stand at room temperature for 10 min. The absorbance at 595 nm was measured against a reagent blank. Bovine serum albumin (BSA) was used as a protein standard.

4.2.18. Statistical Analysis. All reported values were represented as the mean ± S.D (n=4). Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA). Statistical significance was ascertained by one-way analysis of variance, followed by Tukey's multiple comparison tests. Values of $p<0.05$ were considered significant.

4.3.RESULTS

4.3.1. Total Phenolic Content. Polyphenols are a structural class of natural organic compounds characterized by multiple phenol structural units. They are mostly secondary metabolites of a great variety of plants and play important roles including UV screens for protection against ionizing irradiation. Therefore, plant-derived phenolic compounds are of interest due to their antioxidant potential.

In this study, gallic acid (a trihydroxybenzoic acid) was used as a standard. It was found that total phenolic content of the extracts, expressed as µg gallic acid equivalent (GAE)/mg dried SF, was affected by the polarity of the extraction solvent, since GAE values decreased in the following order: hot water > cold water/homogenization > methanol >ethanol > acetone > acetonitrile (Table 4.1). Hot water appears to be the best extraction solvent for polyphenols with the total phenolic content of hot water extract being 12.9 ± 0.17µg gallic acid equivalent / mg of dried SF.

Table 4.1. Total Phenolic, Flavonoid Content and Reducing Power of Tested SF Extracts. At least three independent experiments were performed.

Extraction Method	Total Phenolic Content(µg GAE/mg)	Total Flavonoid Content(µg QE/mg)	Total Reducing Power(µg AAE/mg)
Hot water	12.9213	28.7207	8.6328
Cold water	11.3923	17.4916	7.7434
Methanol	9.2578	24.6844	7.1000
Ethanol	4.6596	17.0028	2.0590
Acetone	2.2935	9.3352	0.9812
Acetonitrile	1.5681	6.0810	0.6779

4.3.2. Total Flavonoid Content. Flavonoids, the derivatives of 2-phenyl-1,4-benzopyrone, are also major plant secondary metabolites. Similar to the polyphenols, flavonoids also fulfill the function of UV filtration in plants, and are found to be the effective component in some herb medicines against LDL oxidation and atherosclerosis due to their antioxidant potential. Therefore, they are also compounds of interest in the current study. Quercetin, a widely-distributed natural flavonol was used as a standard. The total flavonoid content was also found to be affected by the polarity of the extraction solvent in the following order: hot water > methanol > cold water/homogenization ≥ ethanol > acetone > acetonitrile (Table 4.1). The total flavonoid content of the hot water extract of SF was 28.7 ± 0.324 µg quercetin equivalent (QE) / mg of dried SF.

4.3.3. Reducing Power. Chemical reducing power is associated with biological antioxidant activity and may serve as an important index of the antioxidant potential. Ascorbic acid, a naturally-occurring reducing agent, was used as a standard. Reducing power characteristics of different solvent extracts of SF were found to decrease in the following order: hot water > cold water/homogenization > methanol > ethanol > acetone > acetonitrile (Table 4.1). Total reducing power of hot water extract was 8.63 µg ascorbic acid equivalent (AAE)/mg of dried SF, only 1.11 times higher than that of cold water-homogenization and 1.21 times higher than methanol extract. In contrast, the values of ethanol, acetone, and acetonitrile were 4.19 times, 8.80 times, and 12.74 times lower than that of hot water extract, respectively.

4.3.4. Radical Scavenging Power. Free radical scavenging power is an important property for evaluation of the protective effects of an antioxidant because of the deleterious effects of reactive free radicals in biological systems. Butylated hydroxytoluene (BHT), a derivative of phenol and a functionally synthetic analogue of Vitamin E that suppresses radical-mediated autoxidation, was used as a standard. As shown in Figure 4.1, the radical scavenging activities of different SF extracts were affected by the extraction solvent, as the values decreased in the following order: hot water > cold water/homogenization > methanol > ethanol ≥ acetone ≥ acetonitrile.

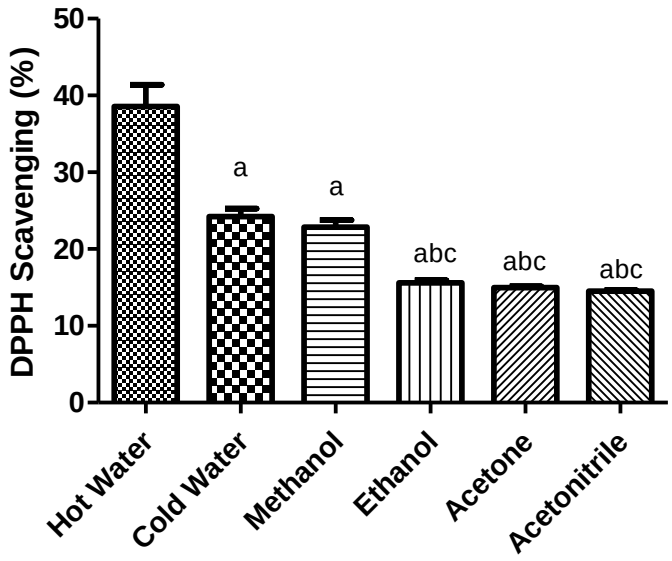

Figure 4.1. Total Radical Scavenging Power of Tested SF Extracts. The absorbance values were converted to the scavenging effect (%) and data plotted as the means of the replicate scavenging effect (%) values ± S.D. (n = 4). The IC_{50} value of the reference compound BHT was 1.82 mg/ml. The concentration of SF extract was 20 mg of dry material/ml. (a: different from hot water extract, b: different from cold water extract, c: different from methanol extract, d: different from ethanol extract, e: different from acetone extract, $p<0.05$).

4.3.5. Hydrogen Peroxide Scavenging Capacity. H_2O_2 is a weakly reactive oxygen species per se; however, due to its neutrality in charge, it is of high cell permeability. Therefore, it enters cells readily and leads to the production of highly active hydroxyl radicals and superoxide radicals in the presence of metal ions. Thus, the scavenging activity for H_2O_2 is an important measure of the antioxidant activity. Sodium pyruvate, which stoichiometrically reacts with H_2O_2, was used as a standard. As shown in Figure 4.2, the H_2O_2 scavenging power of extracts was: hot water > cold water/homogenization > methanol > ethanol ≥ acetone > acetonitrile.

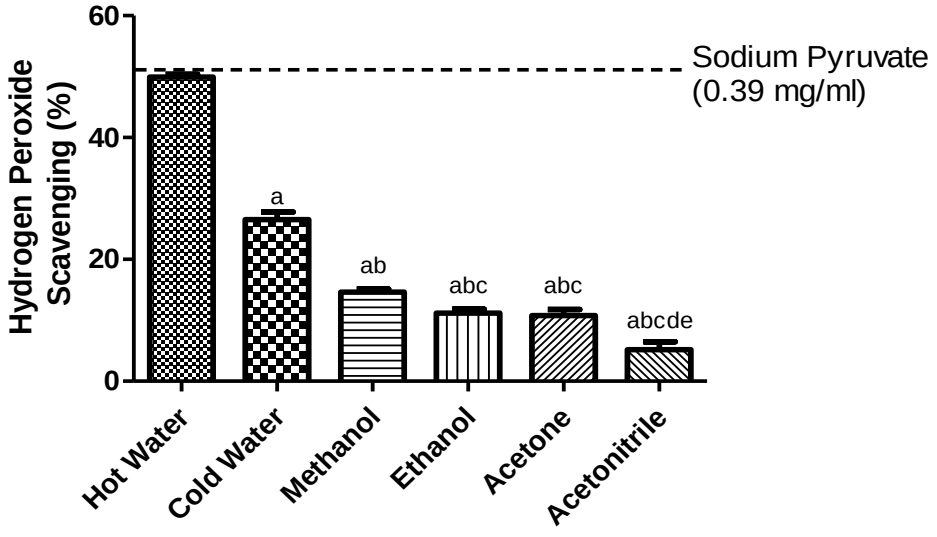

Figure 4.2. Hydrogen Peroxide Scavenging Power of Tested SF Extracts. The absorbance values were converted to the scavenging effect (%) and data plotted as the means of the replicate scavenging effect (%) values ± S.D. (n = 4). The IC_{50} value of the reference compound sodium pyruvate was 0.39 mg/ml. The concentration of SF extract was 20 mg of dry material/ml. (a: different from hot water extract, b: different from cold water extract, c: different from methanol extract, d: different from ethanol extract, e: different from acetone extract, $p<0.05$).

4.3.6. Hydroxyl Radical Scavenging Capacity. The hydroxyl radical is a short-lived (with half-life of~10^{-9} s), highly-reactive free radical that tends to attack nearby biomolecules immediately upon its generation. Of the many different ways by which ˙OH radicals can be produced, the most important is the Fenton reaction, which involves the transition metal catalyzed decomposition of hydrogen peroxide to produce hydroxyl radicals [97]. Mannitol, a polyol which readily reacts with an ˙OH radical, was used as a standard. As shown in Figure 4.3, the SF extracts tested were found to have hydroxyl radical scavenging capacities that decreased in the following order: hot water > cold water/homogenization > methanol > ethanol > acetone > acetonitrile.

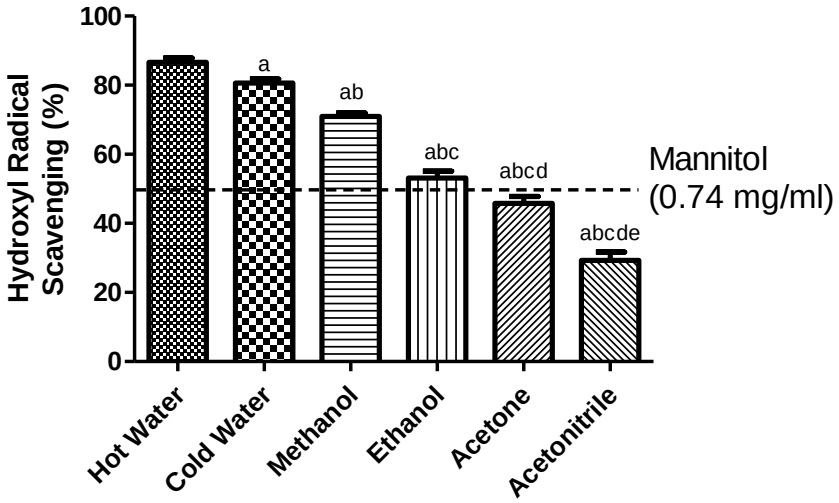

Figure 4.3. Hydroxyl Radical Scavenging Power of Tested SF Extracts. The absorbance values were converted to the scavenging effect (%) and data plotted as the means of the replicate scavenging effect (%) values ± S.D. (n = 4). The IC_{50} value of the reference compound mannitol was 0.74 mg/ml. The concentration of SF extract was 20 mg of dry material/ml. (a: different from hot water extract, b: different from cold water extract, c: different from methanol extract, d: different from ethanol extract, e: different from acetone extract, $p<0.05$).

4.3.7. Superoxide Radical Anion Scavenging Capacity. Superoxide radical anion ($O_2^{\cdot -}$) originates from the one-electron reduction of free molecular oxygen, and is implicated in a number of oxidative stress-related disorders. This is due to its ability to induce the peroxidation of lipids. In this study, quercetin was used as a standard. As shown in Figure 3.4, the superoxide scavenging activities of the tested SF extracts were found to decrease in the following order (Figure 4.4): hot water > cold water/homogenization >methanol > ethanol ≥acetone > acetonitrile.

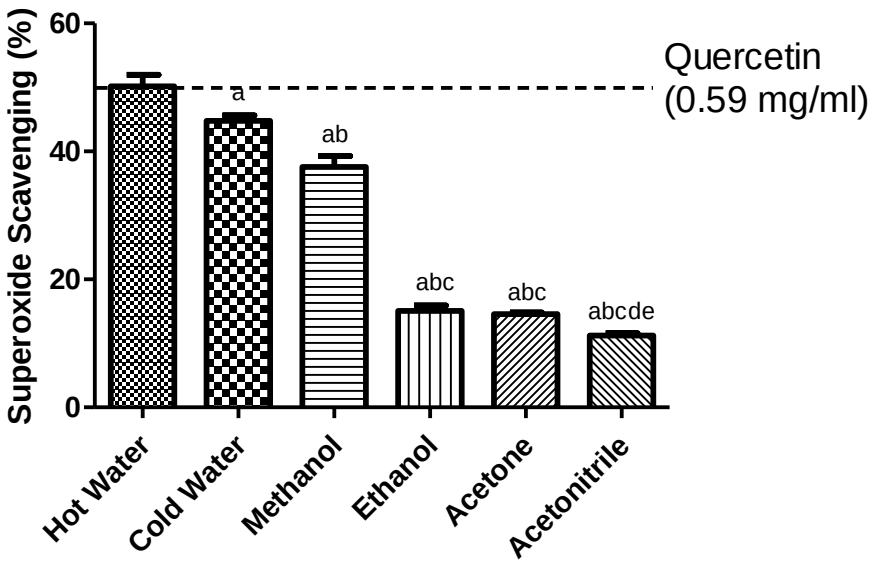

Figure 4.4. Superoxide Scavenging Power of Tested SF Extracts. The absorbance values were converted to the scavenging effect (%) and data plotted as the means of the replicate scavenging effect (%) values ± S.D. (n = 4). The IC_{50} value of the reference compound quercetin was 0.59 mg/ml. The concentration of SF extract was 20 mg of dry material/ml. (a: different from hot water extract, b: different from cold water extract, c: different from methanol extract, d: different from ethanol extract, e: different from acetone extract, $p<0.05$).

4.3.8. Nitric Oxide Scavenging Capacity. NO is a short-lived (half-life 3–30 s) lipophillic colorless gas that can very easily diffuse between cells. Physiologically it functions as an important signal-transduction molecule for vasodilation. However, excessive NO can react with oxygen to produce stable [98] upon reaction with a superoxide [99] which, in turn, can be deleterious. It also plays a role in reperfusion injury. In this study, curcumin was used as a standard. As shown in Figure 4.5, the NO scavenging ability of the extracts followed this order (from high to low): hot water > cold water/homogenization ≥ methanol ≥ ethanol > acetone > acetonitrile.

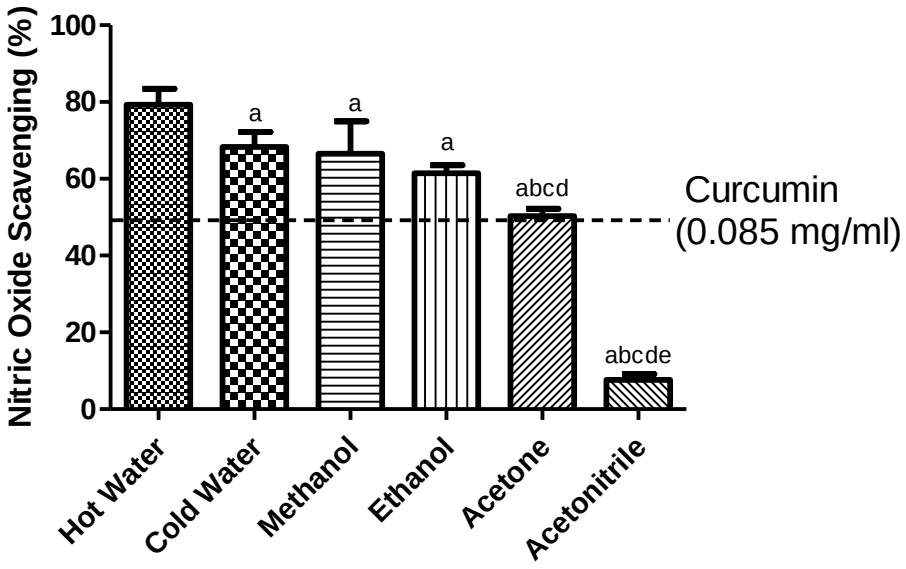

Figure 4.5. Nitric Oxide Scavenging Power of Tested SF Extracts. The absorbance values were converted to the scavenging effect (%) and data plotted as the means of the replicate scavenging effect (%) values ± S.D. (n = 4). The IC_{50} value of the reference compound curcumin was 0.085 mg/ml. The concentration of SF extract was 20 mg of dry material/ml. (a: different from hot water extract, b: different from cold water extract, c: different from methanol extract, d: different from ethanol extract, e: different from acetone extract, $p<0.05$).

4.3.9. Iron - Chelating Capacity. Metal chelating property is especially important because of the ability of transition metal ions to catalyze Fenton reaction. Hydroxyl radical produced as a result of this reaction can accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can abstract hydrogen, propagate the chain reaction [100], and damage cell membranes. Metal chelating activity is, therefore, an important indicator of the antioxidant capacity of a compound. In this study, EDTA was used as standard. As shown in Figure 6, the iron chelating ability was in the following order: hot water > cold water/homogenization >methanol > ethanol >acetone > acetonitrile (Figure 4.6).

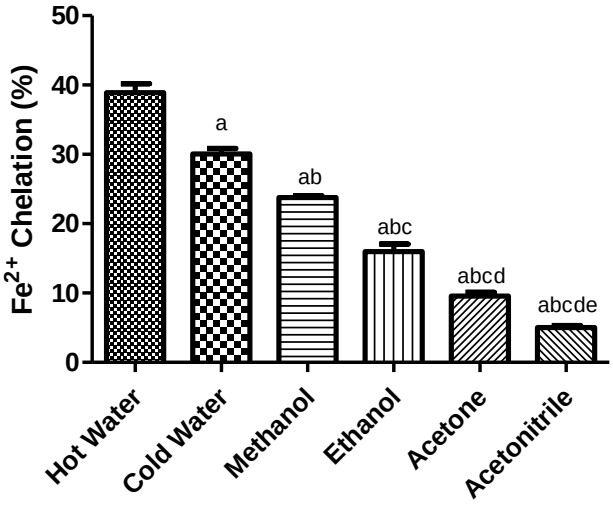

Figure 4.6. Iron Chelation Capacity of Tested SF Extracts. The absorbance values were converted to the scavenging effect (%) and data plotted as the means of the replicate chelating effect (%) values ± S.D. (n = 4). The IC_{50} value of the reference compound EDTA was 0.129 mg/ml. The concentration of SF extract was 20 mg of dry material/ml. (a: different from hot water extract, b: different from cold water extract, c: different from methanol extract, d: different from ethanol extract, e: different from acetone extract, $p<0.05$).

4.3.10. Cytotoxicity of SFE on A549, HepaRG, and CHO Cells. Based on previous results, it appeared that SF hot water extract had the highest antioxidant potential. Therefore, it was chosen for use in subsequent cell-based studies.

As shown in Figure 4.7, SFE was not toxic to CHO cells or A549 cells with a concentration of 500 µg/mL for up to 24 hours. However, a decrease in cell viability was observed in HepaRG cells with SF extract (SFE) treatment above 100 µg/mL for 24 hours. This was confirmed using a calcein AM assay.

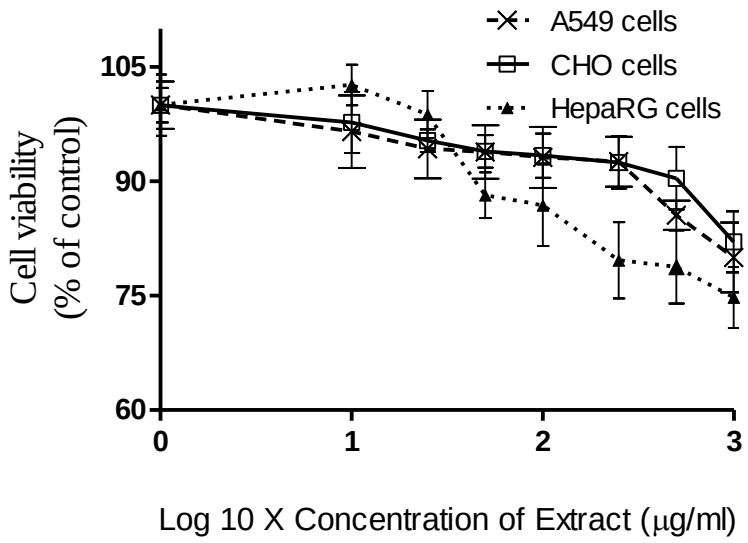

Figure 4.7. SFE Dose-Dependent Toxicity in A549, CHO and HepaRG Cells. Treatment of SFE showed dose-dependent toxicity in all three cell lines. However, at low concentrations (below 10µg/mL) the toxicity of SFE was negligible. At least three independent experiments were performed.

4.3.11. Effect of SFE on t-BHP-Induced Cytotoxicity. To study the protective effects of SFE on t-BHP-induced toxicity, cells were pretreated with various concentrations of SFE for 2 hours, followed by incubation with 50 µM of t-BHP for 24 hours. This resulted in about 40% cell death alone. As shown in Figure 4.8, viability in all three cell lines decreased to approximately 40-50 % of the control, when treated with t-BHP; however, it increased significantly to 500 µg/mL in a dose-dependent manner upon pretreatment with SFE. No further increase was observed upon increasing the concentration to 1000 µg/mL, possibly due to the toxicity of SFE.

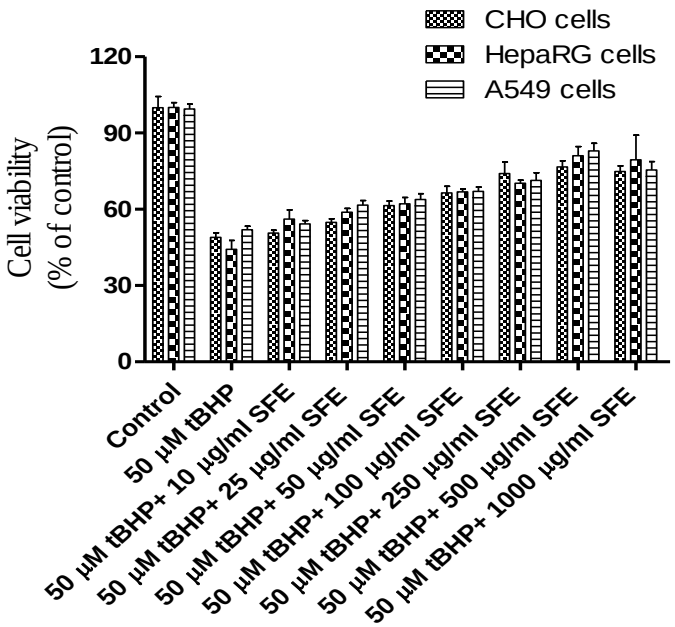

Figure 4.8. Dose-Dependent Protective Effect of SFE on t-BHP-Stressed Cells. Treatment of t-BHP alone decreased cell viability to ~50% of control level in all three cell lines. Pretreatment of SFE prevented cell death in a dose-dependent manner, up to 1000 µg/ml. At least three independent experiments were performed.

4.3.12. Effect of SFE on Intracellular ROS Levels. A dose-dependent increase in the production of ROS was observed in all three cell lines with exposure to tBHP (data not shown). To study the protective effects of SFE on a tBHP- induced increase in ROS levels, all three cell lines were pretreated with various concentrations of SFE for 2 hrs, followed by incubation with 50 µM of tBHP for 2 hrs. As shown in Figure 4.9, there was a significant dose-dependent decrease in levels of ROS with increases in the concentration of SFE, indicating the ROS-scavenging role of SFE.

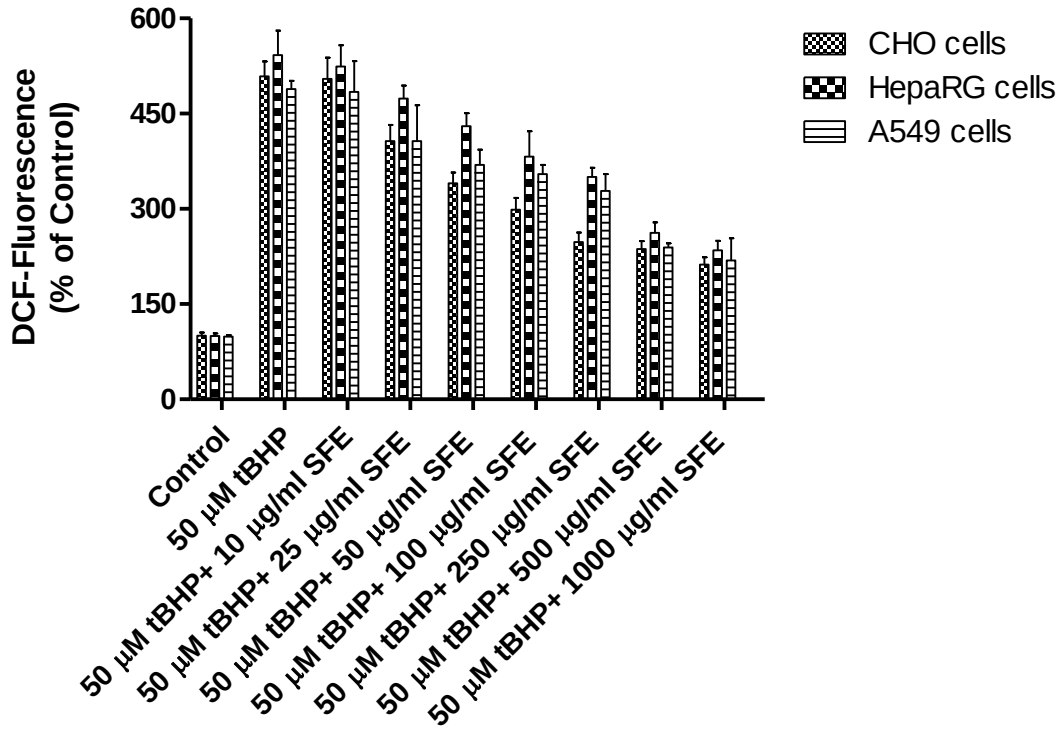

Figure 4.9. Dose-Dependent Intracellular ROS Scavenging Power of SFE on t-BHP Stressed Cells. Treatment with t-BHP alone significantly increased intracellular ROS in all three cell lines, whereas pretreatment with SFE prevented generation of excessive ROS in dose-dependent manner. At least three independent experiments were performed.

4.3.13. Effect of SFE on Intracellular Glutathione Levels. To examine whether SFE acts as an antioxidant by scavenging ROS (thereby preventing further GSH depletion), the levels of intracellular GSH was measured. As shown in Figure 4.10, a 24-hr exposure with 50 μM of tBHP decreased the GSH levels in all three cell lines by more than 50 % of that of the control. Pretreatment with 500 μg/mL of SFE, however, significantly increased the GSH levels in all three cell lines, further verifying the hypothesis about the antioxidant role of SFE.

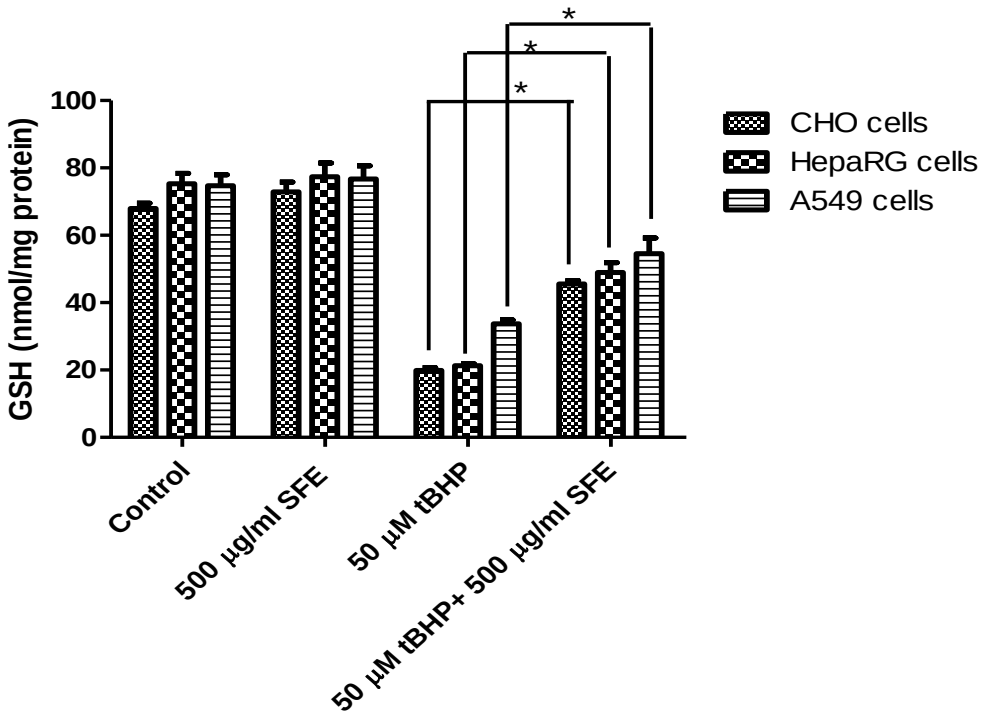

Figure 4.10. Role of SFE on Intracellular GSH Level in t-BHP Stressed Cells. Treatment with t-BHP alone depleted intracellular GSH in all three cell lines, whereas pretreatment with SFE partially restored GSH level. Treatment with SFE alone did not significantly alter the GSH level. At least three independent experiments were performed.

4.3.14. Effect of SFE on Glutathione Disulfide Levels and GSH/GSSG Ratio.

As shown in Figure 4.11, the GSH/GSSG ratios decreased significantly in all three cell lines upon treatment with 50 μM t-BHP; however, pretreatment with 500 μg/mL of SFE restored the ratios of GSH/GSSG in all cells to approximately that of the control group.

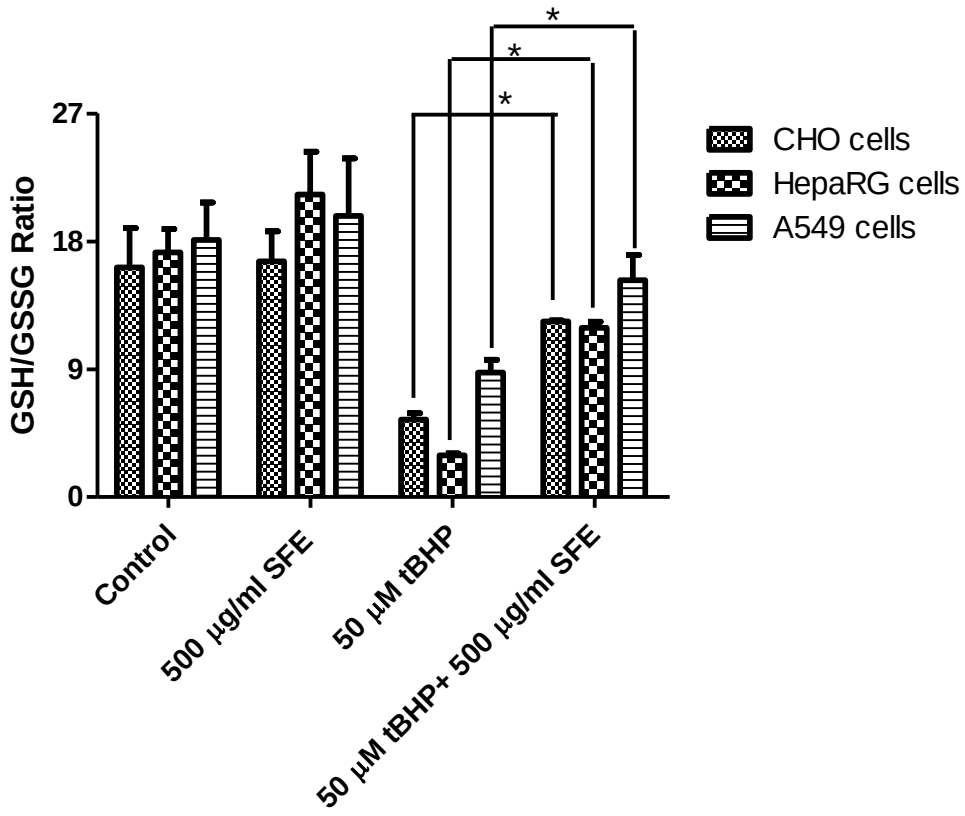

Figure 4.11. Effect of SFE on GSH/GSSG Ratio in t-BHP Stressed Cells. GSH/GSSG ratio decreased dramatically in all three cell lines upon t-BHP treatment. Pretreatment of SFE partially reversed the trends. At least three independent experiments were performed.

4.4. DISCUSSION

Previous studies have been made of the antioxidant role of SF [72][76][101]; however, a comprehensive study comparing the antioxidant potential of different solvent extracts of SF in a cell-free system and in cell lines has not been reported. Here is reported the antioxidant potential of SF extracts for three cell lines, as well as their protective role in t-BHP-induced oxidative stress.

As discussed in Section 3.4., the extracting solvent significantly affected the total phenolic content, flavonoid content, reducing power, and the radical scavenging activities of SF extracts. The yield of the SF hot water extract was about 5% (weight of lyophilized extract/weight of dried plant leaves). Polarity, as well as the temperature of the solvent, affected the antioxidant potential of the extracts, and hot water was found to be the best solvent for extracting total phenolics, including flavonoids. The results were similar to reports of a previous study, in which the antibacterial and antioxidant activities of SF extracts *(*using two different extraction schemes) were investigated. It was reported that the more polar extract had substantial radical scavenging activity, which was attributed to the polar phenolic compounds [72]. In contrast, other researchers reported the highest radical scavenging activity in the methanol, ethyl acetate, and 1-butanol extracts, and the lowest in the aqueous extract. This was attributed to the origin of the plant sample and not the solvent polarity. Their semi-quantitative TLC tests showed smaller amounts of phenolic components in these more polar extracts [101].

The results above indicated that SF plant extracts contained significant amounts of flavonoids, which exerted their antioxidant effect via scavenging or chelation [102][103]. It appeared that the hot water extract had the highest radical scavenging

power, and was more powerful than the BHT standard, considering that the actual yield of the hot water extract was 5% (20 mg yield for dried plant material and 1 mg for the lyophilized hot water extract). However, it did not show good reducing power and it appears that the superior radical scavenging capacity of the hot water extract might account for its significant antioxidant properties.

Superoxide anion, together with its dismutation product, hydrogen peroxide, is deleterious to macromolecules. [104] Flavonoids are found to have an anti-inflammatory effect and to prevent LDL oxidation [105], as well as atherosclerosis[106]. Results of a previous study revealed that these effects were due to the scavenging capacity of superoxide anions and thereby provided protection against oxidative damage [107]. The results suggested that the SF extract is a potent scavenger of superoxide radicals and hydroxyl radicals. Hydroxyl radicals are one of the major reactive oxygen species produced by Fenton's reaction, causing lipid peroxidation, DNA strand cleavage, and subsequent cellular damage [97]. The hot water plant extract proved to be the most potent scavenger of hydroxyl radicals, followed by the superoxide anion radical and then hydrogen peroxide. However, it did not exhibit considerable nitric oxide scavenging ability. Results above concur with a previous publication, which reported that extracts from hot water demonstrated superoxide and hydrogen peroxide scavenging activity, which attributed to the antioxidant activity of the SF hot water extract to the phenolic compounds [76].

Iron can stimulate lipid peroxidation via the Fenton reaction and also by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can further propagate the chain reaction [98]. According to the results, the SF extract was not as

potent a chelating agent as the standard EDTA and, therefore, its antioxidant potential could be primarily attributed to its radical scavenging power. Differences in the antioxidant potential of extracts of SF in different solvents may have been due to the varying composition of the extracts.

The SFE were not toxic to CHO and A549 cells below a concentration of 500 µg/mL. However, mild toxicity was observed in HepaRG cells above100 µg/mL, which could have been due to differences in the cells and their susceptibility to SFE. SFE protected against t-BHP-induced oxidative stress and increased cell viability in all the three cell lines in a dose-dependent manner, up to 0.5 mg/mL

However, no further increase in cell viability was observed above this concentration, possibly due to the toxicity of SFE itself. Results above were in accordance with previous research, which reported that SF hot water extract, up to concentrations of 40 µg/mL, had no adverse effect on the viability of human neutrophils after a 30 min treatment [76]. Results from present study were also supported by another study on proximal and distal convoluted tubule epithelial cell lines (LLC-PK1 and MDBK), in which the cell viability of both cell lines treated with concentrations between 6 mg/mL and 0.3 mg/mL for 48 h was more than 89% [103]. Results from another study about toxicity of SFE were similar to the results where it was shown that, although high concentrations of a SF extract (ethanol) can be toxic to normal T cells, SFW (water) fractions were relatively non-toxic. It was found that 0.5 mg/mL SFW extract showed 81% live cells after 24 hrs [105]. In addition, safety studies in vervet monkeys and humans have suggested that SF extracts are not toxic [106, 108].

In contrast, some studies have shown cytostatic and cytotoxic effects of SF extracts in cervical carcinoma cells, Chinese hamster ovary cancer cells, Caski and Jurkat T Lymphoma cells, human breast adenocarcinoma (MCF-7), human non-tumorigenic epithelial mammary gland cells (MCF-12A), MDA-MB-468 cell line, human leukemia Jurkat cells, human promyelocyte HL60 cells, MDA-MB-231 breast cancer cells, DU-145 prostate cancer cells, and proximal and distal convoluted tubule epithelial cell lines (LLC-PK1 and MDBK) [74, 103, 109-111].

These contrasting results could be due to a variety of factors such as innate differences in cell lines, strategy of preparation of extracts (tablets vs. dried plant parts), the extraction solvent, dosage and time of administration, as well as varying components in plants grown in regions with different soil compositions and environmental factors (leading to synthesis and accumulation of secondary metabolites) [112].

It was shown that SFE protected cells by scavenging ROS in a dose-dependent manner in all three cell lines. These results are also in line with a previous report that the SF hot water extract significantly decreased both the luminal and lucigenin enhanced chemiluminescence responses of neutrophils stimulated by FMLP in a dose-related manner [76].

To further elucidate the mechanism of protection against t-BHP-induced oxidative stress, GSH and GSSG levels were measured. GSH, in its reduced form, was the most powerful intracellular antioxidant, and the ratio of reduced to oxidized glutathione (GSH/GSSG) was representative of the oxidative status of the cell. An increase in ROS, together with a decrease in GSH, set off a cascade of further oxidative damage. SFE was able to prevent depletion of GSH in all three cell lines. Results of present study were

supported by a study done by Ngcobo [113] where the SF extracts decreased both cell viability and GSH levels in H9 cancerous cells, while the same extracts significantly increased cell viability and GSH levels in normal T cells. The extracts caused a time-dependent decrease in GSH content in H9 cells with the SF water extract dilutions being more effective than the ethanol extracts. However, in normal T cells, the extracts negatively affected the levels of GSH at higher concentrations, but enhanced the GSH content at lower concentrations. The SF water extract dilutions were also more effective in increasing the GSH content of normal T cells than the ethanol extracts were. However, contrary to this, a significant decrease in GSH was reported in SF-treated MDBK cells and LLC-PK1 cells [103]. These contrary results could, again, be due to differences in cell lines, doses, and incubation times (considering the cytotoxicity of SFE at high concentrations), as well as extract preparation.

4.5. CONCLUSION

The results indicated that hot water is an ideal solvent for the extraction of the antioxidant ingredients of SF vegetative material. Flavonoids may be the key component responsible for the antioxidant potential of SF based on its superior radical scavenging ability. In addition, protection against t-BHP-induced oxidative stress for transformed as well as for normal cell lines further demonstrates its antioxidant potential. *In vitro* assays indicated that this plant extract is a significant source of natural antioxidants, which might be helpful in preventing the progression of various oxidative stresses.

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